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RECENT STUDIES IN THE MECHANISMS OF EMBRYONIC DEVELOPMENT

BY

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RECENT STUDIES IN THE MECHANISMS OF EMBRYONIC DEVELOPMENT*

Consulting Editor: Roberts Rugh

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OOPLASMIC SEGREGATION IN RELATION TO DIFFERENTIATION

By DONALD PAUL COSTELLO
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OF the many processes which take place during the maturation, fertilization, and early cleavage of invertebrate eggs, it is apparent that some are more significant than others in causally contributing to differentiation. The fact of parthenogenesis indicates that the contributions of the spermatozoon to development, particularly its nuclear contribution, cannot be of primary significance. The fact that certain ova, under experimental conditions, may show various degrees of differentiation without cleavage, indicates that neither the mitotic mechanism, nor cleavage, nor the cleavage pattern, nor cell boundaries are of essential importance. It is likewise clear, from experimental evidence as well as by genetic assumption, that the differential competence of the different blastomeres of the early embryo cannot be accounted for on the basis of nuclear, chromosomal, or genic differences between the blastomeres. We are, thus, led to the conclusion that the factors necessary for differentiation are those producing the localization of the cytoplasmic areas of specific potency—or, if one wishes to use another terminology, the factors producing cytoplasmic fields and gradients. The study of such factors may be approached from the standpoint of metabolic measurements, in terms of differential metabolic rates and enzyme distribution. However, I believe that a more direct *biological* approach to the problem is afforded by the study of the process that has been termed oöplasmic segregation.

The segregation of the visible cytoplasmic elements in the eggs of marine invertebrates was early described by Wilson (1892), Conklin (1905), Lillie (1906), and others, and has been more recently studied by Spek (1930, 1934a, 1938) under the term, *bipolar differentiation*. It is the purpose of the present paper to review the recorded facts in relation to oöplasmic segregation and to make some suggestions concerning the significance of the process.

The phenomenon of visible oöplasmic segregation is particularly striking in eggs showing the so-called "determinate" type of cleavage, and may be initiated, in different forms, at the time of, or prior to, germinal vesicle breakdown, during polar body formation, or at fertilization or parthenogenetic activation. Invertebrate eggs of the various animal groups may be divided into different categories as regards the time of onset, and the pattern, of oöplasmic segregation. There are also different relationships between the pattern of segregation and the cleavage

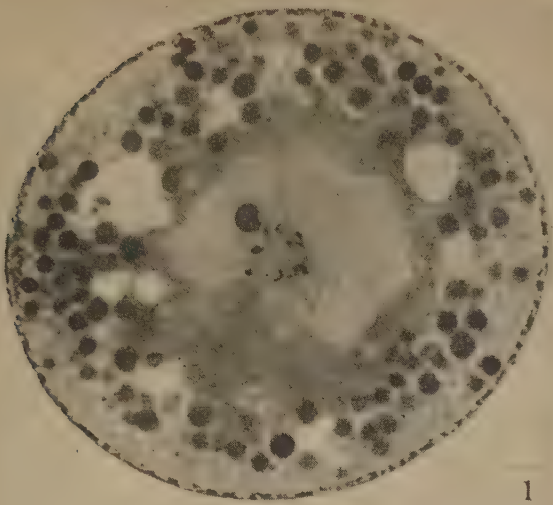
52609

pattern. Because of these various types of segregation patterns, Spek's term, bipolar differentiation, is a misnomer. Since there is an original polarity of the egg that can be traced back to the earliest stages of the oögonium, it is recognized that the simplest type of oöplasmic segregation is essentially a re-polarization of certain of the egg constituents.

Oöplasmic Segregation in the Egg of NEREIS. The bipolar pattern of oöplasmic segregation is, perhaps, best exemplified by the *Nereis* egg. Spek (1930) studied the process in the egg of *Nereis dumerilii*, using vital staining methods. The process of segregation of the oöplasmic constituents does not begin until after extrusion of the polar bodies (FIGURES 1 and 2). It is especially apparent at the four-cell stage, as figured by Spek (1934a) for *Nereis limbata*, where the animal hemisphere gives an alkaline reaction with indicator dyes and the vegetal hemisphere gives an acid one. This has been discussed in considerable detail by Costello (1945a) for the egg of *Nereis limbata*. Since the animal hemisphere is destined to give rise in development to the ectodermal quartets of micromeres, whereas the vegetal hemisphere produces the endodermal macromeres, the implication of Spek's work is that there is a causal relationship between this "bipolar segregation" of acid and alkaline "protoplasm" and the differentiation of endoderm and ectoderm. I shall return later to the discussion of this point.

In normal eggs of *Nereis*, the time of onset of visible oöplasmic segregation is shortly after the final incorporation of the sperm head into the egg. Since an accentuated aggregation of cytoplasmic components is obtained in *Nereis* eggs from which the activating spermatozoon is removed (along with the vitelline membrane) by alkaline sodium chloride (Costello, 1945b), sperm entrance is not a necessary prerequisite. Certain salt solutions (Spek, 1930, 1934b) may also induce segregation in unfertilized eggs.

Oöplasmic Segregation in the Egg of CHAETOPTERUS. In the egg of *Chaetopterus*, segregation takes place much earlier than in that of *Nereis*, and it is essentially completed by the metaphase of the first maturation division (Lillie, 1906). The ectoplasm of the ovarian egg covers the free hemisphere and ends a short distance below the equator, so that the endoplasm comes to the surface in the vegetal hemisphere. There is usually also a small ectoplasmic defect at the animal pole, where the endoplasm comes to the surface. After the egg is shed by the female *Chaetopterus* into sea water, the germinal vesicle ruptures and a series of movements of egg substance takes place. The ectoplasm flows toward the vegetal pole, covering the exposed endoplasm. The original polar defect enlarges and the maturation spindle becomes attached there. There is also a redistribution of the different types of endoplasmic granules *a*, *b*, and *c* (see Lillie, 1906, Figures 1, 2, 5, and 25). This distribution of substances corresponds, in many respects, to the future embryonic areas and is maintained from this time on. At the first cleavage, the polar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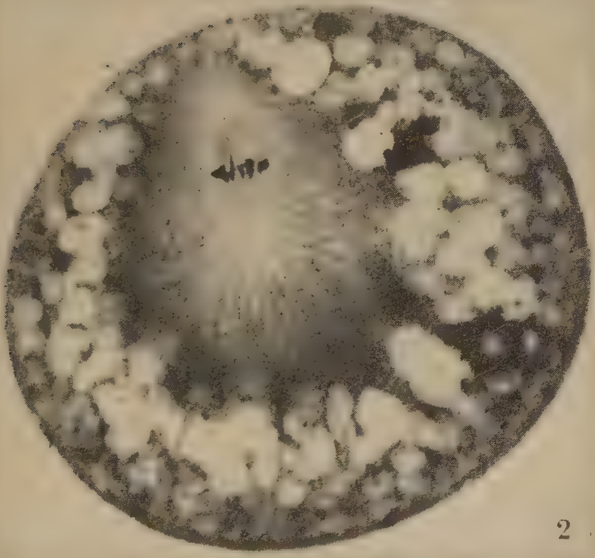
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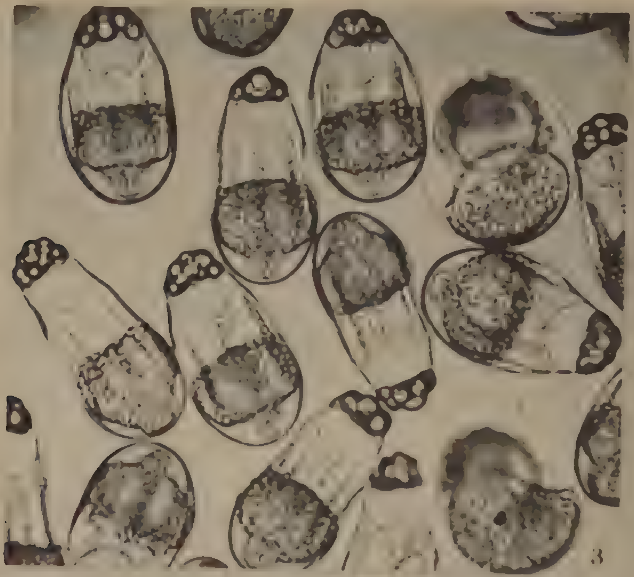
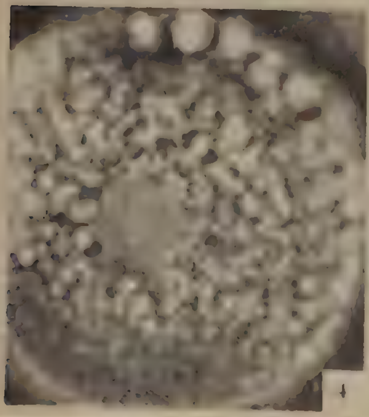
Figure 1. Section of fertilized egg of *Nereis limbata*, at prophase of first maturation division. Note chromosomes and nucleolus in rupturing germinal vesicle, and concentric orientation of oöplasmic inclusions as in unfertilized egg. Photomicrograph, magnification 525x.

Figure 2. Section of fertilized egg of *Nereis limbata*, at metaphase of first maturation division. The oil and yolk have been dissolved by the reagents. There has been no segregation of inclusions other than that brought about by movement of the spindle toward the animal pole. Presumably, the spindle is formed by rearrangement of the structural proteins of the protoplasm. Photomicrograph, magnification 590x.

bodies and ectoplasmic defect are transmitted to the *CD* blastomere, and at the third cleavage the polar defect is found on the 1*d* blastomere, which gives rise to the apical tuft. A more detailed account of the be-





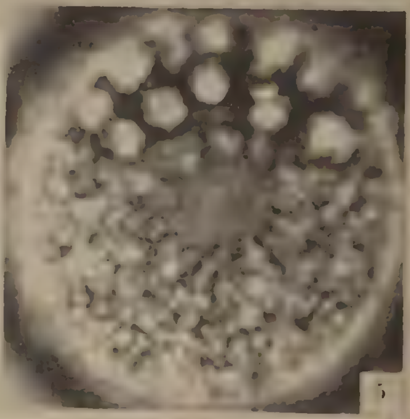


FIGURE 3. Unfertilized *Nereis* eggs centrifuged 60 minutes at about 6000 times gravity, photographed 5 minutes after centrifuging. The large oil droplets mark the centripetal end. All inclusions are sedimented into strata. Magnification 135x.

FIGURE 4. *Nereis* egg centrifuged 24 minutes in 0.73 M sucrose at 6000 times gravity, inseminated immediately, and photographed 53 minutes later. Note oil droplets marking centripetal pole. Polar area, in surface view, is at equator. Magnification 110x.

FIGURE 5. *Nereis* egg centrifuged 10 minutes in 0.73 M sucrose at 6000 times gravity, inseminated immediately, and photographed 70 minutes later. Note oil droplets, marking centripetal pole, at top, polar area and first polar body in surface view at equator. Magnification 110x.

havior of the *Chaetopterus* egg during this segregation period is given by Lillie (1906) and summarized with special reference to the possible role of the polar defect, by Costello (1945a).

The Mechanism of Oöplasmic Segregation. With these brief descriptions of two examples of the process of oöplasmic segregation, we have materials for a consideration of the mechanism involved. Spek (1930, 1934a, 1938) speaks of the process as being brought about by a "self-cataphoresis," but there is no evidence for a flow of electric current through the cell, and no evidence that such a distribution of substances is obtained when a potential difference is superimposed upon the cell from outside. Nor will the action of gravity (or magnified gravity, as in a centrifuge) in sedimenting the contained oöplasmic inclusions produce a similar pattern. For example, in oöplasmic segregation in the *Nereis* egg, oil and yolk go toward the vegetal pole, whereas in the centrifuge (FIGURE 3), oil moves centripetally, yolk centrifugally, to opposite directions, which are usually at right angles to the animal-vegetal axis (FIGURES 4 and 5). This is due to orientation of the somewhat disc-shaped egg in the centrifuge, with polar axis (short axis of the disc) at right angles to the direction of the centrifugal force.

An explanation of oöplasmic segregation was recently proposed (Costello, 1945a) in terms of the Teorell "diffusion effect." As illustrated in FIGURE 6, the Teorell system is as follows: Across a permeable bound-

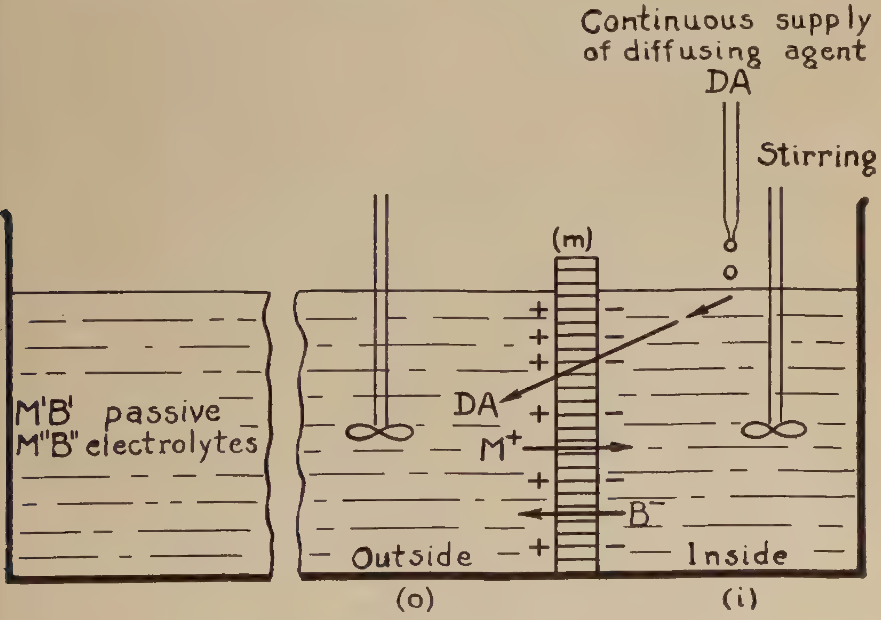


FIGURE 6. Diagram to illustrate Teorell's experimental arrangement for demonstration of the "diffusion effect." (After TEORELL, 1937.)

ary (m) there is assumed to be present a constant difference in concentration of either the cation D^+ or the anion A^-. The maintenance of this condition is accomplished by the continuous addition of the substance DA to the small volume (i) [= inside], causing DA to act as a "diffusion agent" which steadily diffuses across (m) into the part (o) [= outside]. In (o), a fixed, constant composition is maintained by keeping the volume large.

The continuous steady diffusion of DA was shown by Teorell (1935a, 1937) to influence the distribution of other electrolytes present, denoted by $M'B'$, $M''B''$, etc., which were not participating in any active diffusion and were therefore called "passive ions." The membrane is permeable to these passive ions. It was assumed that the D^+ ions had a higher mobility in the boundary (m) than the A^- ions, producing an electrical potential across the boundary. Starting with the initial state of equal concentrations of M^+ and of B^- on the two sides of the membrane (m), the electrical potential causes an inward migration of M^+ and an outward migration of B^-. Finally the concentration gradients become sufficiently large to balance the electrical gradient and the system approaches a steady state. Thus, the M^+ ions accumulate and B^- ions decrease in amount inside. Therefore, a diffusion of one electrolyte may produce, inside, an accumulation or impoverishment of other cations or anions, depending upon the mobilities of the ions of the diffusing agent. This effect upon ionic distribution is called the diffusion effect.

Teorell's concept of diffusion effect was later extended (1935b) to apply to any electrically charged particle, regardless of size. The diffusion potentials present in the system would thus move positively charged particles into the negative part of the diffusion potential field, and negatively charged particles in the opposite direction. It was emphasized that this "diffusion effect" upon ionic and colloidal distribution is not a cataphoretic effect, because no current is flowing and no external E.M.F. is applied. It is brought about by exchange of charged particles due to differences in mobility of the ions of the diffusing substance within the membrane.* Teorell (1935c) also devised a scheme for studying ionic distribution within a thick diffusion layer—the so-called multi-membrane arrangement.

In my 1945 paper, I proposed a biological analogue of the Teorell scheme to account for oöplasmic segregation (FIGURE 7). If there were a continuous supply of diffusing substance entering the cell across a special area of the cell membrane, such as the polar area, diffusion potentials might be set up within the egg, with part of the protoplasm (such as the protein framework) acting as a multimembrane. If electrically charged particles, regardless of their size, were subjected to the influence of these diffusion potentials, positively charged particles would be moved toward one pole of the egg, negatively charged particles toward the other. In this earlier paper, I considered at some length the assumptions basic to the application of the Teorell scheme in such a case, and the reader is referred there for further details. I should like to point out that Harrison (1945) has recently visualized the ovum (Harrison's Text figure 12) in a manner essentially corresponding to my last figure, with a protein framework and distribution within this framework of two types of substances. Harrison distinguishes two types of polarity in the ovum: a polarity of direction (Richtungspolarität) and a polarity of stratification (Schichtungspolarität). The former is equivalent to what I have called the original polarity of the egg, the latter to what is brought about by the process of segregation.

---

* The normal difference in mobility of the ions of a substance is sufficient, but this mobility difference may be accentuated by certain types of membranes (Teorell, 1937).

If oöplasmic segregation is initiated when the egg enters sea water, or when some event, such as fertilization, alters the membrane permeability to permit entry of the diffusing substance, then the diffusing substance is probably either one of the common constituents of sea water, or a constituent of the outer surface of the egg which is able to enter the egg after its permeability is altered. Spek (1930, 1934b) has induced an artificial "bipolar differentiation" in the eggs of *Nereis* and *Asterias* by means of potassium chloride. Mead (1898) induced *Chaetopterus* eggs to complete their maturation by the addition of a small quantity of potassium chloride to sea water. It is also this substance which induces *Chaetopterus* eggs to undergo differentiation without cleavage (Lillie, 1902, 1906). There is, therefore, the possibility that the diffusing substance may be potassium chloride. However, it is very important

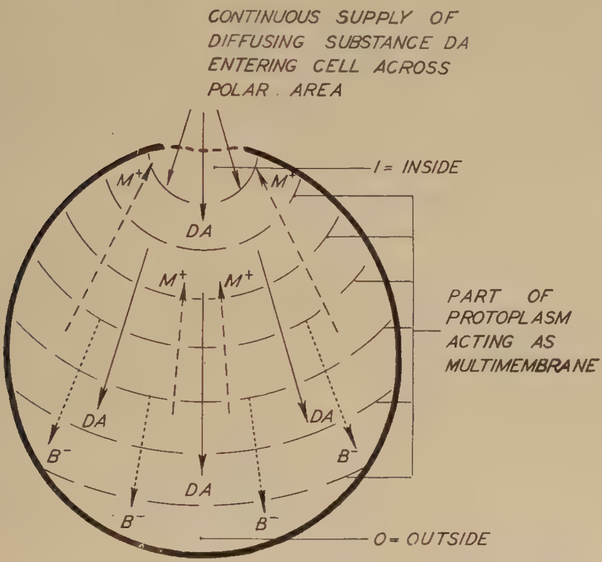

CONTINUOUS SUPPLY OF DIFFUSING SUBSTANCE DA ENTERING CELL ACROSS POLAR AREA
I = INSIDE
M+
M+
DA
M+
M+
PART OF PROTOPLASM ACTING AS MULTIMEMBRANE
DA
DA
DA
B−
B−
B−
B−
O = OUTSIDE

FIGURE 7. Biological analogue of Teorell scheme, employing "multimembrane" or thick diffusion layer to bring about segregation of particles M+, B− within cell. (After COSTELLO, 1945a.)

that the diffusing substance should show a considerable difference in mobility of its ions within the "membrane" [*i.e.*, (m)], in order to set up a diffusion potential of any great magnitude. The mobilities of the potassium and of the chloride ion are almost the same in simple solutions. We do not know with certainty what their relative mobilities would be within the egg protoplasm.

It is a well-known fact that modification of the external medium, as by the addition of a simple salt such as lithium chloride, leads to modification of development. Some examples of lithium-induced exogastrulation in the sea urchin, *Arbacia punctulata*, are shown in FIGURES 8–13.*

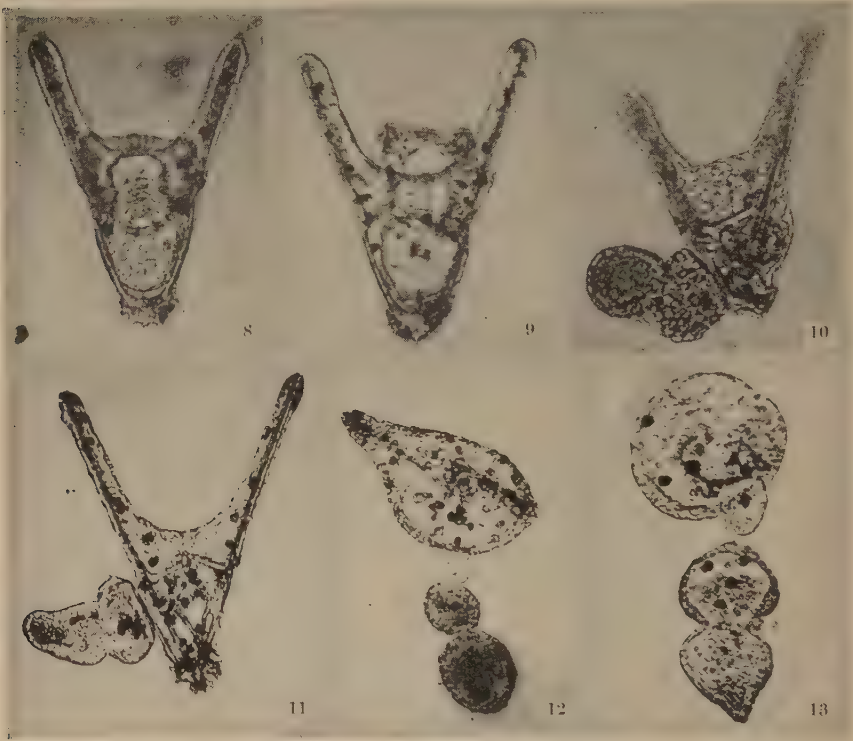


Figure 8. Normal, living pluteus of *Arbacia punctulata* about 40 hours old. Viewed from aboral surface, magnification 150x.

Figure 9. Same, viewed in median optical section.

Figure 10. Exogastrulated pluteus, 48 hours old, developed from *Arbacia* egg treated for 4 hours with mixture of 0.54 M LiCl (20 parts) and sea-water (80 parts), beginning 53 minutes after insemination (during 2-cell stage). Magnification 150x.

Figure 11. Exogastrulated pluteus, 52 hours old, same treatment as FIGURE 10. Magnification 130x.

Figure 12. Exogastrulated pluteus, 57 hours old, same treatment and magnification as FIGURE 10. Effect more extreme.

Figure 13. Exogastrulated pluteus, 52 hours old, same treatment and magnification as FIGURE 12. Effect still more extreme.

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* Observations made by Mrs. Marjorie Hopkins Fox and the author in 1945.

It is possible that the lithium acts through an effect on the diffusion potential and oöplasmic segregation. *Arbacia* embryos from a given culture which has been subjected to a specific dosage of lithium chloride to induce exogastrulation, exhibit a remarkable variation in the degree of developmental modification. Child (1940) has exhaustively discussed this variation in susceptibility to lithium. He suggests that aggregation at the bottom of the container produces environmental conditions different from those surrounding isolated individuals. One might argue that an internal protoplasmic gradient would tend to produce just such a high degree of variability if the eggs come to rest with their polar axes oriented at any angle with respect to the bottom of the dish. That is, in some cases, the external chemical gradient effect would be added to the original polar gradient; in other cases, the two would act in opposite directions; and in still other cases, at all possible angles to each other.

Since the early experiments of Herbst (1892), it has been recognized that lithium has at least two distinct effects on echinoderm eggs. It produces exogastrulation (*i.e.*, separation of endodermal from ectodermal and mesodermal structures with essentially complete differentiation of all three) and inhibition of development, which may produce either inhibitory ectodermal or endodermal modifications. Perhaps a third effect is endodermalization of prospective ectoderm. If we can attribute these separate effects to different physico-chemical causes, I should postulate that the primary exogastrulation is brought about by a physical effect of the lithium ion through some such mechanism as the Teorell diffusion effect acting on oöplasmic segregation, accentuating the separation of ectodermal and endodermal factors responsible for differentiation. The secondary inhibition of development could be assumed to be due to an inhibitory effect of lithium on certain enzyme systems (however, contrast Pease, 1942).

There are a number of unexplained effects of certain other external agents in embryonic development. As a working hypothesis, it might be possible to invoke the Teorell diffusion effect as the causative mechanism and plan experiments to further elucidate these phenomena. For example, the effect of blood externally applied when inducing parthenogenesis in unfertilized frog's eggs by pricking needs further investigation. It is well known (Bataillon, 1912) that the presence of blood materially increases the percentage of haploid embryos reaching an advanced developmental stage. Tyler (1931) has described radially symmetrical parthenogenetic embryos of *Urechis*. He suggests that these received a diffuse activating stimulus, rather than a stimulus from one side (such as that provided in normal fertilization), and leading to formation of a bilaterally symmetrical embryo. Tyler (1941) suggests that parthenogenetic activating agents would best be applied in the form of a gradient. In line with these suggestions, we might postulate that the presence of the blood modifies the diffusion gradients set up through the point of puncture of the pricked frog's eggs, or modulates the stimulus to produce more normal embryos. One possible experimental test of this hypothesis is the study of the action, under similar circumstances, of various large molecules with isoelectric points near those of the several blood components.

Utilization of the Teorell diffusion effect as an explanation of the mechanism of localization of certain substances within the ovum does not necessarily imply that the diffusion agent enters the cell from outside. A diffusion gradient of substances leaving the cell, or diffusing from one region to another within the protoplasm, could produce a similar effect. The original polarization of the ovum, laid down during the early growth stages in the ovary, presumably sets up a polar difference in metabolically significant substances. This is one of the primary tenets of the axial gradient theory of C. M. Child. The products directly or indirectly resulting from these differences in metabolic activity may be free to diffuse from regions of higher to those of lower concentration. In so diffusing, under certain conditions, a Teorell diffusion effect may be established, thus secondarily inducing a movement of other charged particles. Gene products, diffusing from the nucleus or chromosomes during certain periods of cell activity, may similarly bring about movements of other substances.

A SPECIAL CASE: *Styela partita*. While the scheme outlined above (in terms of a polar defect) might account for segregation of materials along the polar axis, it could not, without addition or modification, account for a more complex type of oöplasmic segregation, such as that found in the egg of *Styela* and described by Conklin (1905). Upon fertilization of this ascidian egg, there is a primary segregation of materials resulting from a downflow of the yellow and clear substances from the animal toward the vegetal pole (Conklin, 1905, Figures 1–6). This active migration is completed within ten minutes after the entrance of the spermatozoon. Then the sperm nucleus moves to one side in the lower hemisphere, inaugurating a secondary segregation of materials to form the posterior yellow crescent (Conklin, 1905, Figures 8, 9, 13, 14, 15). Opposite this crescent, at the future anterior region of the egg, the light gray crescent arises. As a result of segregation, the animal hemisphere is occupied by clear protoplasm and the remainder of the vegetal hemisphere by dark gray yolk. In line with the polar defect theory outlined above, it would be tempting to suggest that the sperm entrance point serves as a second point of entrance for a diffusing substance which sets up the secondary bilateral pattern of the embryo, but such an explanation is inadequate. As Conklin (1905) demonstrated, the sperm nucleus does not always take the shortest path to the equator, but appears to move in a certain meridian. This seems to indicate that the path of the spermatozoon is determined by the structure of the cytoplasm. We must, thus, assume a bilateral orientation predelineated in the "framework" of the ground substance. Therefore, Harrison's (1945) diagram of the pattern of the ovum must be modified to include bilaterality as well as polarity, in order to be applicable to the case of the unfertilized *Styela* egg. The situation obtaining in the unfertilized *Styela* egg may perhaps best be visualized in terms of a bilateral liquid crystal structure extending throughout the cell (FIGURE 14), serving as the "framework" of the protoplasm, and leading to a directed diffusion. If directed diffusion leads to the establishment of a particular pattern of oöplasmic segregation, perhaps we may dispense entirely with the polar defect hypothesis. It is probable that segregation takes place in both the polar and antipolar fragments of cut *Cerebratulus* eggs. The antipolar fragment lacks the polar defect. Possibly, a liquid crystal structure with polar and bilateral orientation provides the requisite conditions.

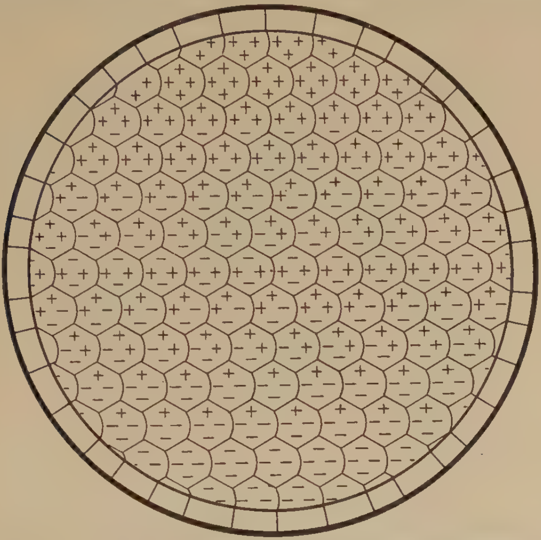

FIGURE 14. Diagram of hypothetical structure of an ovum, modifying HARRISON'S (1945) text figure 12 to include a cortex and a basic pattern of bilaterality in the lattice of the ground-substance. The lattice also provides a structural arrangement for the polarity of direction, while the distribution of the two kinds of particles (designated by + and −) within the interstices of the lattice indicates the polarity of stratification.

Undoubtedly, different patterns exist in the unfertilized eggs of different species of animals. In the frog's egg, it is probable that bilaterality is not determined until the time of fertilization. In the eggs of ascidians, it is apparently predetermined in the unfertilized egg. In the eggs of mollusks and annelids showing spiral cleavage, bilaterality may be determined at the time of fertilization (Just, 1912; Morgan and Tyler, 1930), but there is presumably an asymmetry of structure leading to spiral cleavage of the egg or its fragments, which is determined at an early precleavage stage.

The Significance of Oöplasmic Segregation. No more striking example of the significance of oöplasmic segregation can be found than in the case of differentiation without cleavage in the egg of *Chaetopterus*. After a short exposure to certain solutions (mixtures of $2\frac{1}{2}$ molar potassium chloride and sea-water) both fertilized and unfertilized eggs pass through certain well-defined phases of segregation, as described by Lillie (1902, 1906) and by Brachet (1937). The yolk accumulates as a dense mass in the interior, and other granules or vacuoles assume a polar or peripheral position. FIGURE 15* shows a photomicrograph of one of these embryos, just before the differentiation of cilia. There is a remarkably clear-cut segregation of parts. Subsequently the peripheral protoplasm becomes ciliated and more vacuolated, so that the embryo resembles, in part at least, a trochophore with inner yolky endoderm, ciliated surface and equatorial band of vacuoles. Lillie's (1902) Figure 8 shows a normal trochophore and his Figures 1–7, etc., show the ciliated structures which develop without cleavage from unfertilized eggs. Embryos differentiating without cleavage do not develop an apical tuft. Lillie correlates this with the fact that the defect in the ectoplasm, where the endoplasm comes to the surface, is obliterated by the artificially induced flowing movements.

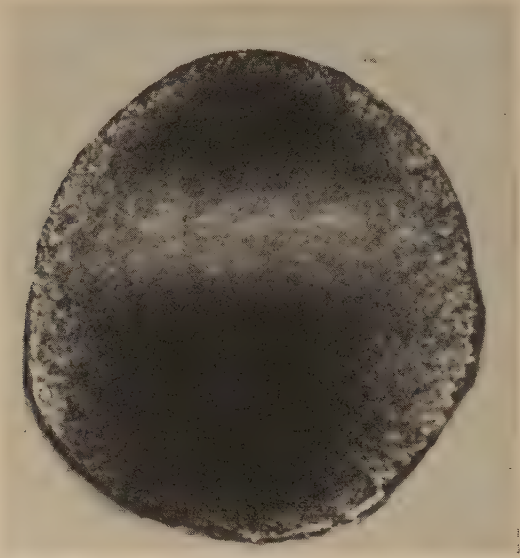

FIGURE 15. Photomicrograph of an unfertilized egg of *Chaetopterus pergamentaceus*, treated with a mixture of 2.5 M potassium chloride (10 parts) and sea-water (90 parts) for 64 minutes. Photographed 3 hours and 45 minutes after treatment. Note marked segregation of cytoplasmic components. A few hours later, cilia were differentiated at the surface. Magnification 660x.

In the same cultures,* some eggs are found in which the streaming movements of the interior protoplasm continue after the initial segregation has been accomplished. These amoeboid masses never differentiate the structures so characteristic of the other pseudo-larvae. Therefore, it appears that the proper degree of segregation must be maintained for differentiation to occur.

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* See footnote on page 670.

It is my belief that oöplasmic segregation is of much greater significance in interpreting the development of egg-fragments than has been suspected previously. Studies of the development of egg-fragments, such as the classical experiments of E. B. Wilson (1904) on the egg of *Dentalium*, clearly indicate an association of specific embryonic potency with certain protoplasmic areas. For example, the lower polar area of the egg of *Dentalium* contains the material of the antipolar lobe. This region is definitely related to the formation of apical tuft and post-trochal regions. In *Dentalium*, there is, thus, a very early prelocalization of embryonic potency, associated with the very early visible segregation of the lower polar area.

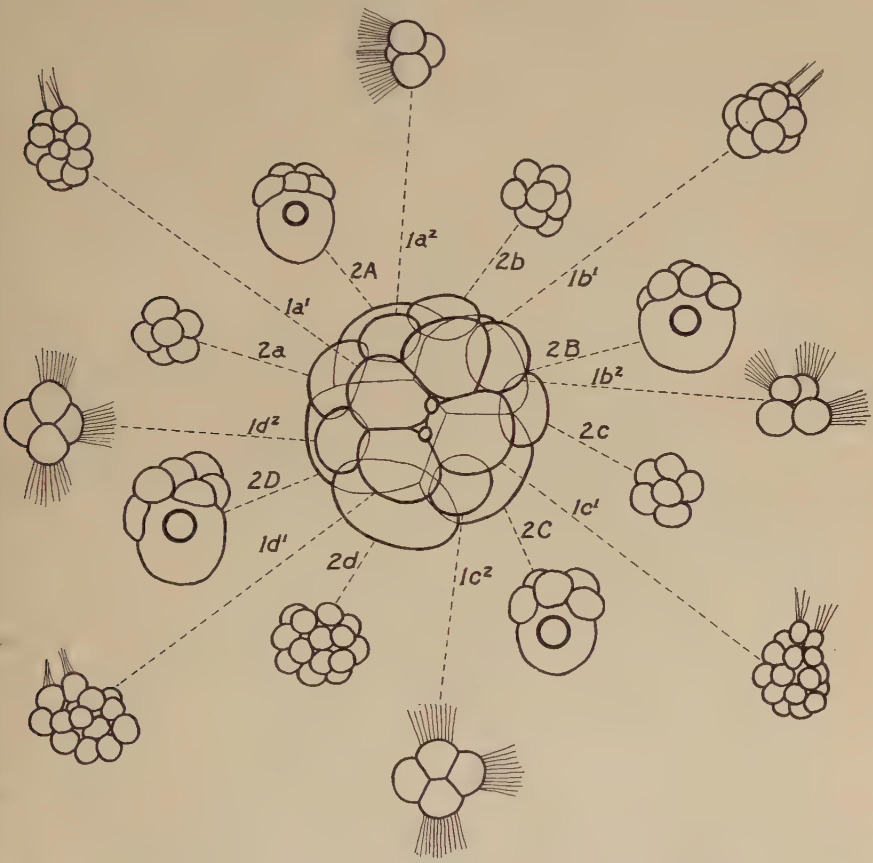

Figure 16. Differentiation of the blastomeres of the 16-cell stage of the *Nereis* egg after separation by dissection. (After Costello, 1945b.)

In the egg of *Nereis limbata*, it has been shown (Costello, 1945b) that, from the time of the first cleavage, the isolated blastomeres develop as partial embryos. This is demonstrated very clearly by isolating the blastomeres of the 16-cell stage (FIGURE 16), when only isolated trochoblasts ($1a^2$–$1d^2$) differentiate prototrochal cilia, only macromeres tend to gastrulate, etc. It is, therefore, of interest to inquire whether this cleavage mosaic is foreshadowed by a prelocalization extending back into the unsegmented egg. Cutting the *Nereis* egg across the equator, shortly before the first cleavage, produces polar fragments (upper fragments) containing the cleavage spindle, and antipolar fragments lacking spindle and nuclear materials. Only the polar fragments cleave (TABLE 1) and may produce essentially complete embryos (Costello, 1940a). Isolated

TABLE 1

DEVELOPMENT OF FRAGMENTS OF FERTILIZED *NEREIS* EGGS OBTAINED BY HORIZONTAL SECTION

	Number							
	Isolated	*Cleaved*	*Ciliated*	*Gastrulae*	*Proto. pigm.*	*Eyespots* *(1)*	*Eyespots* *(2)*	*Anal pigm.*
Nucleated (polar)	25	20 (+3 abn.)	16	16	4 (+1?)	1 (+1?)		1
Non-nucleated (antipolar)	25	0	0	0	0	0		0
Whole denuded eggs	35	35	35	+	12	6	6	6

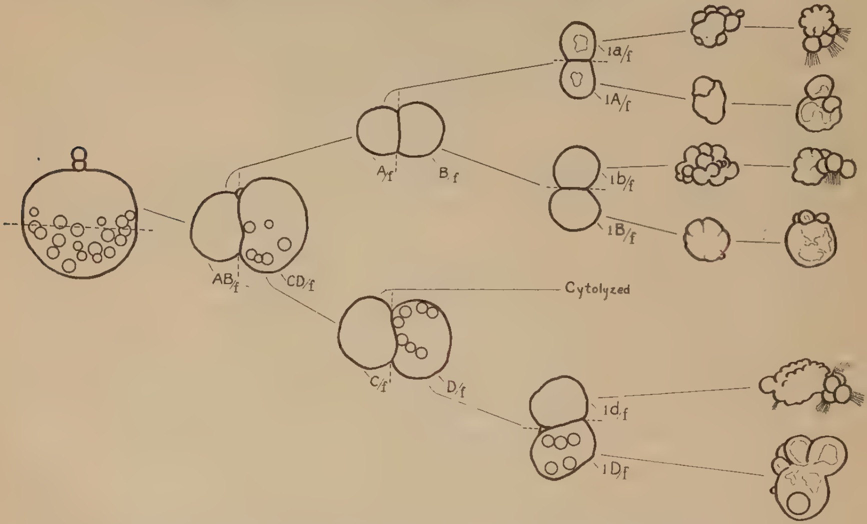

FIGURE 17. *Camera lucida* sketches of progressive blastomere separation of the cleavage products of an egg fragment of *Nereis*. The row of figures at the extreme right indicates the final products of differentiation of the surviving isolates. Compare with progressive blastomere separation of whole egg (COSTELLO, 1945b, Figure 4).

blastomeres of these egg-fragments develop in essentially the same manner as isolated blastomeres of whole eggs (FIGURE 17), clearly indicating that the prototrochal material cannot be at the extreme lower pole of the fragment, but occupies a position corresponding to its position in the whole egg (Costello, 1940b). Originally, I suggested three alternative hypotheses (FIGURE 18) to account for this result. First (FIGURE 18, a), it is possible that there is no prelocalization of the materials for prototroch formation (/) and for gastrulation (\) just prior to the first cleavage, when the egg-fragments were obtained. Secondly (FIGURE 18, b), it is possible that these materials are already segregated, but are segregated in the animal hemisphere, the lower half of the egg being essentially unimportant at this time. Thirdly (FIGURE 18, c), it is possible that the segregation of potencies has already occurred, but that the animal fragment is capable of regulating and produces the missing potencies out of other materials than those originally destined for these parts. I am now inclined to view the first hypothesis as the most likely. Since visible oöplasmic segregation in *Nereis* does not begin until after the formation of the polar bodies and is not well-advanced until just before the third cleavage, it is reasonable to suppose that germinal prelocalization is occurring simultaneously with visible segregation of the formed cytoplasmic inclusions.

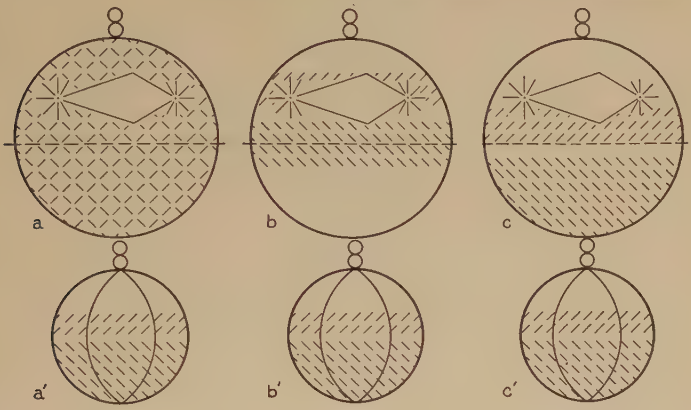

FIGURE 18. Diagrams of three alternative hypotheses concerning distribution of materials in whole eggs and egg-fragments (see text).

If a series of different egg species were studied, I believe that it would be possible to establish a correlation between the time of oöplasmic segregation and the degree of embryonic determination. However, we must bear in mind that centrifuging experiments on many forms have clearly indicated that there is no causal relation between visible particles, displaceable with centrifugal force, and morphogenetic values. For example, some of Spek's beautiful figures of *Nereis* eggs stained with neutral red and nile blue sulfate show a striking "bipolar differentiation." My own studies have shown that, when these stained *Nereis* eggs are subjected to an appropriate centrifugal force (FIGURE 19), all stained materials are sedimented into definite strata. Such eggs may nevertheless develop normally. The "acid" and "alkaline" regions are not regions of acid or alkaline ground substance (hyaline protoplasm) but are regions in which the contained granules or vacuoles stain differentially. So far as we can ascertain by observation of stained and unstained eggs, there are no visible differences in the *hyaline protoplasm* of the different regions.

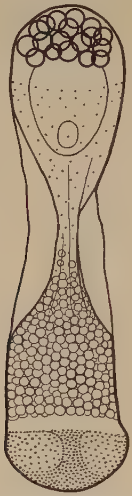

FIGURE 19. *Nereis* egg centrifuged 61 minutes at 66,000 times gravity. Sketched immediately, before change of shape. The strata, in order, proceeding from the centripetal pole, are: oil layer; hyaline zone surrounding upper portion of germinal vesicle; indistinct layer of fine granules, which stain with neutral red; broad stratum of yolk spheres; a second, narrow hyaline zone; centrifugal zone of heavy granules, including vortex of granules which stain deeply with neutral red.

The conclusion that the visible cytoplasmic components of invertebrate eggs have no morphogenetic value has recently been questioned by Raven (1938) on the basis of experiments on the eggs of *Nereis* and *Chaetopterus*. Raven and Bretschneider (1942), using low centrifugal forces on the eggs of *Limnaea*, have objected to the conclusions of Conklin (1910) and Clement (1938). However, their objections appear to me to be without adequate foundation, since they ignore the fact that Clement (1938) obtained normal development of *hyaline fragments* of centrifuged *Physa* eggs. Harvey (1946) recently obtained plutei from the clear quarter of the *Arbacia* egg.

The apparent paradox between the results on the development of egg-fragments and of centrifuged eggs admits of easy solution. We need only assume that the mechanism of normal segregation segregates both *visible* formed inclusions and *invisible* morphogenetic substances. The invisible morphogenetic substances, such as hormones, enzymes, or the like, become associated with the hyaline protoplasmic base in the interstices between the granules or vacuoles. Subsequent centrifuging might easily displace the large visible particles without displacing the invisible substances which are associated with the protein framework of the cell. This conclusion is basically the same as that of Conklin (1931) resulting from centrifugation of the *Styela* egg. It is not the visible granules that have morphogenetic value, but the special, localized hyaline protoplasm with which these granules are normally associated.

The Teorell diffusion effect theoretically provides us with a mechanism which might bring about both a visible stratification of the suspended oöplasmic substances and a parallel but invisible segregation of the "formative stuffs." The primary requirement is that both types of materials be charged.

It has been suggested by Weiss (1939) that diffusible substances (such as inductors or hormones) can act only to bring out differences already existing in an embryo. He uses as an analogy the photographic developer which does not create the picture, but merely converts a latent image into a visible one. Weiss states (pp. 441–442), "It is inconceivable that a chemical agent diffusing indiscriminately through a body whose parts are all alike should ever be able to produce local differences. . . . the problem of organization can expect no elucidation from the study of the 'dark-room' phase of the process." I should like to point out here that a diffusible substance which possesses a difference of mobility of its dissociable parts could act, *via* the Teorell effect, to bring about a *segregation* of other charged particles and thereby actually create a pattern where none previously existed.* Two diffusion gradients of different types, originating from points 90 degrees apart, might give a more complex pattern. An interaction between the components of this pattern, once a certain threshold of concentration has been reached, might produce still more complex patterns.

Another example of a pattern which can be produced by diffusion of a substance into a gel lacking a preformed structural basis for the pattern is the well-known Liesegang phenomenon of colloid chemistry. When two solutions, capable of forming a relatively insoluble precipitate, are allowed to interact inside a gel, the precipitate, under certain conditions, is deposited in layers which are repeated in regular fashion. These are the Liesegang rings, or Liesegang stratifications. The explanation of this phenomenon, which involves diffusion in relation to the degree of local supersaturation, can be found in any good textbook of colloid chemistry.

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* Obviously, diffusion from one side, or directed diffusion, is not *indiscriminate* diffusion. But one could visualize a pattern of segregation arising if an egg rests with one surface against the substratum, inhibiting diffusion from this side, and permitting free diffusion into the free surface from the external medium (*vide* work on the *Fucus* egg, summarized by Whitaker, 1940). The diffusion of the amphibian organizer from the chorda-mesoderm into the overlying ectoderm is no more indiscriminate. Organizer action, which appears to depend upon the competence of the reacting tissue, is probably a typical example of "developer action" similar to that described by Weiss for hormones.

In view of the lack of data on the physico-chemical conditions within the developing ovum, the diffusion effect theory of oöplasmic segregation is, at present, only a working hypothesis. I have suggested this hypothesis, not in the belief that it explains all the facts, but rather in the hope that a more adequate explanation of oöplasmic segregation may be evolved. It is hoped, furthermore, that this presentation may rekindle interest in the classical materials of experimental embryology, the eggs of the marine invertebrates.

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Discussion of the Paper

DR. ROBERT CHAMBERS (*New York University, New York, N. Y.*):

I was interested in Dr. Costello's introducing a diffusion gradient model as a hypothesis to explain orientation in an egg. I only wish he could have taken time to present more, and in greater detail, from the great wealth of his own experimental observations.

Concerning the existence of a polar differentiation which persists from the ovarian egg, I wish to mention the egg of the *Cerebratulus*. The excentrically placed germinal vesicle maintains the same relative position as that of the nucleus of the cells of the germinal epithelium, while the micropyle, which is an interruption of the vitelline membrane, as it expands on contact with sea-water, lies at the opposite pole where the epithelial cell had separated from its substrate. Hence, under normal conditions and in the absence of external disturbing factors, the polar bodies arise almost exactly opposite the spot where the sperm is most likely to enter. Insemination can occur anywhere over the surface of the egg, but the growing aster, once the sperm has entered, brings the sperm-head into the position where nature had originally intended it to be. Can it be that the gelating monaster, with the streaming entailed in its growth, has something to do with a reorientation of previously dislocated basic patterns? We know that the development of totipotent egg-fragments is also preceded by the formation of a monaster. May not this phenomenon produce the required polar orientation in each fragment?

Dr. D. P. Costello:

Theoretically, it seems possible, as Dr. Chambers suggests, that the streaming movements associated with the growth of the sperm aster may orient substances within the egg, to give a polarity of stratification, though not a polarity of direction. The latter is probably laid down in the egg during its earliest oögonial history. However, if we postulate this activity as a characteristic of sperm asters, we encounter the difficulty of explaining orientations which bear no constant relation to the position of the sperm path, as has been pointed out for the case of the secondary bilateral segregation in the egg of *Styela*. Rashevsky has suggested the possibility that the centrioles of asters are diffusion centers, bringing about an orientation of chromosomes in the mitotic figure, but there is no direct evidence for this. He has not discussed centrioles in relation to oöplasmic segregation.

Dr. A. M. Shanes (*New York University, College of Dentistry, New York, N. Y.**):

Dr. Costello has suggested that Teorell's theoretical approach to the "diffusion effect," devised to account for ionic gradients, is applicable to the distribution and movement of particles in the fertilized egg. It may be desirable to call attention to a limitation of the theory as developed by Teorell and its possible significance.

Teorell assumes that diffusion potentials set up by the continuous diffusion of ionized substances are responsible, in the steady state, for the differential distribution of ions on either side of any boundary which serves to support such diffusion gradients. Unfortunately, under conditions of rather high electrolyte concentration such as exist within most if not all cells, such diffusion potentials would be insignificant. Thus, Dr. Osterhout set up a model in which CO_2 served as a source of diffusing ions across a non-aqueous layer. A differential distribution of ions occurred, but the expected potentials arose as ionic changes proceeded rather than before. The potentials were obviously the result rather than the cause of the ionic transfer, and this even though hydrogen ions (the most mobile and therefore the best from the standpoint of large diffusion potentials) were involved and their gradient very high.

Dr. Osterhout's results are easily interpreted from the standpoint of the Donnan equilibrium, for bicarbonate ions were retained within the "cell" while hydrogen ions exchanged with other "extracellular" small cations. Thus bicarbonate constituted the indiffusible ions required for a Donnan equilibrium; the ionic gradients and associated potentials would thus develop as actually observed.

It is difficult to see, in view of such results, how a diffusion effect could cause the movement of microscopically visible particles, particularly with the speed and over distances as great as those which have been described for some eggs. Even if molecules as small as proteins were concerned, the porosity of the protoplasm necessary for their migration would eliminate the possibility of ion retention such as seems to be required for the diffusion effect.

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THE ORGANIZATION OF THE AMPHIBIAN EGG DURING FERTILIZATION AND CLEAVAGE

By G. FANKHAUSER
Princeton University, Princeton, N. J.

Introduction

IN contrast to the beautifully transparent or semi-transparent eggs of invertebrates and ascidians reviewed by Costello, where processes of oöplasmic segregation may be followed under the microscope, the eggs of amphibians are perfectly opaque. Following fertilization, one can detect sufficient changes at the egg surface to be sure that more important rearrangements of materials must be taking place inside. However, when the egg is fixed and sectioned, one merely sees a heavy suspension of yolk platelets, with a small amount of cytoplasm scattered among these presumably inert materials. There is little to attract our immediate attention and to arouse our curiosity.

It is no wonder that the study of the organization of the amphibian egg in fertilization and cleavage was long neglected. Well in the foreground during the early days of *Entwicklungsmechanik*, in the hands of Born, Schultze, Roux, and Spemann, it was pushed into the background by the sensational success of the transplantation method that may be applied to the egg in slightly more advanced stages. During the past few years, there has been a revival of interest in the initial stages of development. Obviously, to trace the origin of the already complex organization at the blastula and gastrula stages is as important a task today as it ever was (*cf.* Harrison, 1945).

The unsatisfactory state of our knowledge of the egg in its earliest stages is vividly portrayed by the fact that the revival of interest at once led to an animated controversy between different workers. Dalcq and Pasteels assume a relatively simple organization of the ovum at fertilization which becomes gradually more complex during cleavage. Lehmann, on the other hand, is convinced that the condition of the unsegmented egg is very similar to that of the beginning gastrula, with little change during segmentation. Although the two viewpoints appear to be fundamentally different, they are likely to converge as more facts become known.

To make matters worse, the professional language of the embryologist is full of ambiguous and, perhaps, outmoded terms that are bound to foster misunderstandings. Several eminent investigators have warned us against the dangers inherent in the use of such expressions as "determination," "segregation," "organ-forming substances," "organizers," etc.

The list of terms "the use of which is not recommended," to borrow Needham's phrase (1942), is growing all the time and may soon include most of those that have become dear to us through long association and, therefore, seem to be full of meaning, although it now appears that this meaning may not be exactly the same for all workers in the field. It seems doubtful that the solution to these problems of vocabulary will be found in the introduction of a whole series of new terms. While some, if generally accepted, may help to clarify, others are bound to add to the confusion. As far as the organization of the ovum is concerned, the primary need is for more and better established facts of sufficient convincing power to bring about more general agreement among different workers on the factual level.

Under these circumstances, it may be most profitable to review the overall picture of the organization of the amphibian ovum as it stands today, to point out the few accomplishments and the great gaps in our knowledge that still exist. Such a review should comprise (*1*) the observations on the visible organization of the egg, including both nuclear and cytoplasmic phenomena; (*2*) the experimental tests of the invisible organization of the cytoplasm; and (*3*) the results of experimental analysis of the nucleus in so far as they concern our problem.

Visible Organization of the Amphibian Egg

Nuclear Phenomena. At the very beginning of its career, the egg of almost all salamanders passes through a crisis which might well prove to be fatal if it were not for the existence of a special compensating mechanism. In frogs and toads, fertilization is normally monospermic. If, under laboratory conditions, two or more spermatozoa are allowed to enter the egg, the supernumerary sperm nuclei divide independently, at the same time as the diploid fusion nucleus. Cleavage is abnormal, leading to the formation of haploid, diploid, and mixed cells, and development comes to an end in embryonic or early larval stages (Brachet, 1910, 1912; Herlant, 1911).

In the majority of urodeles, on the other hand, fertilization is normally polyspermic. Up to ten or more spermatozoa may enter the egg, depending on the species. During the first three hours, the internal developments are similar to those in polyspermic frog's eggs and seem to predict certain disaster. However, at the critical time, as the principal sperm nucleus unites with the egg nucleus, the accessory sperm nuclei begin to show signs of degeneration. They may go on to prophase and even release the chromosomes, but the sperm asters do not divide, and the remnants of the accessory nuclei are soon pushed out of the way by the large asters of the diploid mitotic system (FIGURE 1; Fankhauser 1932a; Fankhauser and Moore, 1941a). The nature of the inhibiting factor is not known. It seems to spread from the vicinity of the dominant nuclear system. In any case, there must be a change in the cytoplasm

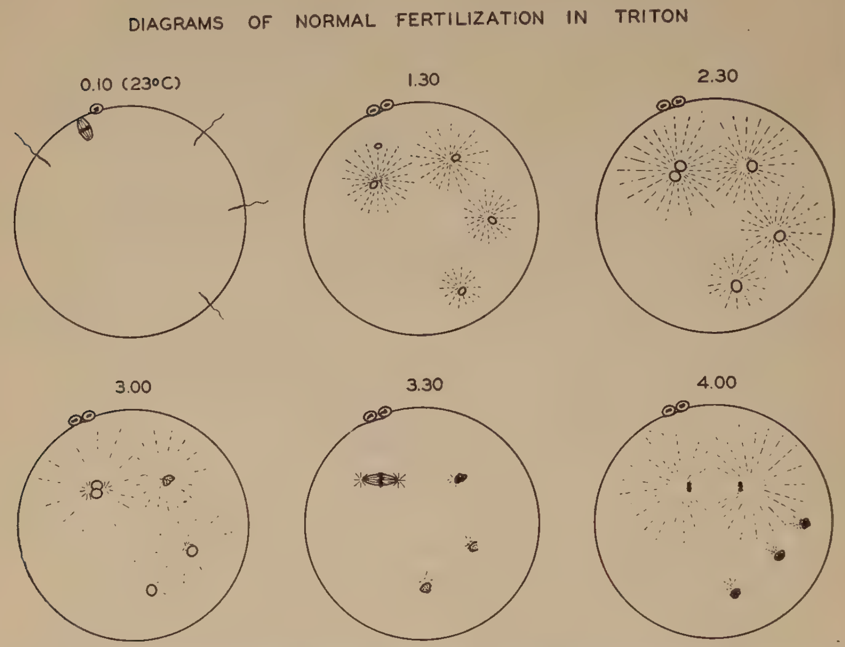

FIGURE 1. Diagrams of polyspermy in the European Newt, *Triton*. The egg is represented in side view, animal pole up; the nuclei, maturation spindle, and cleavage spindle at metaphase are greatly enlarged relative to the size of the whole egg. The asters are not enlarged.

10 minutes after insemination (at 23° C.): metaphase of second maturation division, penetration of four spermatozoa.

1 hour 30 minutes: second polar body given off; small egg nucleus moves toward nearest sperm nucleus which will become the principal sperm nucleus. All accessory sperm nuclei develop normally. Differences in the size of the sperm asters are related to the amount of active cytoplasm present in the animal and vegetal hemispheres.

2 hours 30 minutes: egg nucleus and principal sperm nucleus in contact. Maximum development of sperm asters. Accessory sperm nuclei still normal.

3 hours: fusion of egg and principal sperm nucleus; fading of sperm asters. Two small asters appear in center of principal sperm aster. Accessory sperm asters remain undivided. Accessory sperm nucleus nearest fusion nucleus shows signs of degeneration.

3 hours 30 minutes: metaphase of first cleavage mitosis. All accessory sperm nuclei degenerating. Note eccentric position of cleavage spindle.

4 hours: early telophase of first cleavage mitosis. Note growth of asters at poles of first cleavage spindle which tends to center the mitotic figure and to push the remnants of accessory sperm nuclei out of the animal hemisphere.

surrounding the accessory sperm complexes which blocks their division.

We easily forget that both nucleus and cytoplasm are also involved in the fulfillment of another prerequisite of normal development, *viz.*, the establishment of a mitotic apparatus that will assure normal cleavage of the egg. Under normal conditions, the cycles of the nuclear and cytoplasmic components of the mitotic system are perfectly coordinated. However, if we prevent the union of the egg nucleus and the principal sperm nucleus, by dividing the fertilized egg into two parts, or by eliminating one of the nuclei, the chromosomal and centrosomal cycles are frequently thrown out of gear. In fragments of salamander eggs, and in whole eggs following the removal of the egg chromosomes, the isolated sperm nuclei show a whole series of abnormal mitotic phenomena: absence or inactivity of the division center leading to a monaster; delayed division of the center; precocious division producing multipolar figures; and, finally, the cytoplasmic component may go through the paces all alone and still be able to induce division of the cell body (FIGURE 2; Fankhauser, 1934a; Fankhauser and Moore, 1941b).

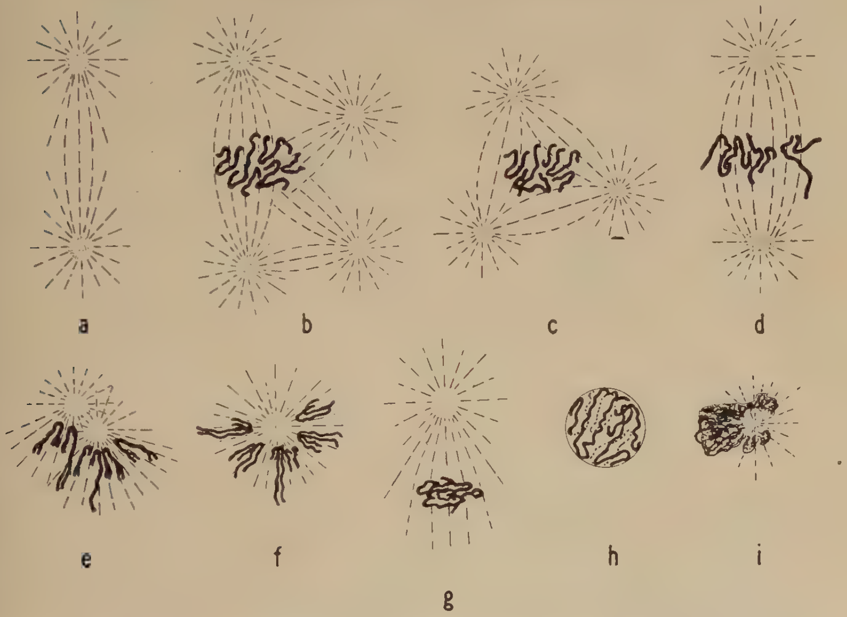
BEHAVIOR OF SPERM NUCLEUS IN ANDROMEROGONY IN *TRITON*

FIGURE 2. Diagrams of various types of mitosis found in androgenetic egg fragments of *Triton*. In the absence of the egg nucleus, accessory sperm nuclei may divide. Frequently, the chromosomal and centrosomal cycles are out of gear. Several types of mitosis may be found in a single egg fragment.

a, division of aster in complete absence of chromosomes.

b and c, precocious division of sperm aster producing tetra- and tripolar figures.

d, normal mitosis.

e, delayed division of aster, at a time when the chromosomes had oriented their attachment points toward the single aster. Whole (split) metaphase chromosomes are distributed irregularly between the two poles.

f, typical monocentric mitosis (monaster); aster remains undivided, chromosomes divide normally.

g, abortive monaster (chromosomes fail to orient their centromeres toward the single pole and do not divide).

h, sperm nucleus in prophase, no aster (later stages of "anastral" mitosis of sperm nuclei have not been found).

i, degeneration of sperm nucleus without attempt at division.

The egg nucleus, when isolated in a fragment of the fertilized egg, is in a more difficult position since it is not normally associated with an active division center. A centrosome may arise, but it usually fails to divide. If no centrosome appears, the egg nucleus may remain in late prophase and grow to extraordinary size, or it may divide repeatedly by an anastral mitosis that looks as if it had been borrowed from a plant cell (FIGURE 3). It is important to note that this anastral form of mitosis also may induce cleavage (Fankhauser, 1937). Cell division itself, the most basic process of development, is an example of a phenomenon that was known, in the days of classical developmental mechanics, under the perhaps inadequate but illustrative name, "multiple assurance."

BEHAVIOR OF EGG NUCLEUS IN GYNOMEROGONY IN *TRITON*

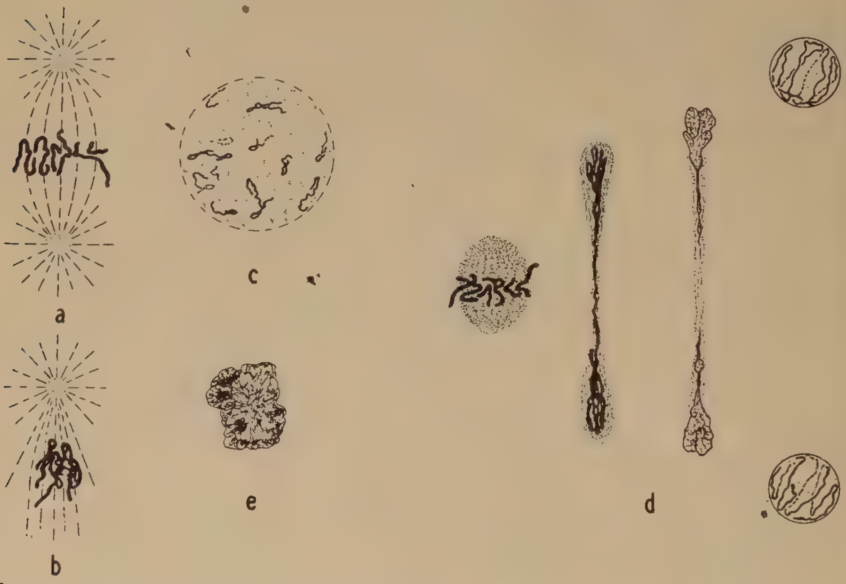

FIGURE 3. Diagrams of types of mitosis in gynogenetic egg fragments of *Triton* (fragments of fertilized eggs containing the egg nucleus alone).

a, normal mitosis of egg nucleus (very rare).

b, abortive monaster.

c, "permanent" prophase with continued swelling of nucleus (twelve chromosomes, the haploid number in *Triton*, may be counted).

d, four stages in anastral mitosis; *metaphase:* with short "spindle" formed by "half-spindle components" derived from nuclear sap; *anaphase:* stretching of spindle material, incomplete separation of chromosomes; *telophase:* disappearance of spindle material (note similarity to amitosis); *prophase* of following mitotic cycle.

e, degeneration of egg nucleus without attempt at division.

In this connection, another observation should be mentioned which clearly shows how particular conditions in the ooplasm may control the behavior of the nucleus. Following A. Brachet's (1922) discovery of the peculiar reactions of the spermatozoa in immature sea urchin eggs, Bataillon (1929, 1934) described similar phenomena in eggs of *Hyla* and *Triton*. In immature eggs which contain the first maturation spindle, the spermatozoa do not form vesicular nuclei associated with large asters. Precociously, small chromosomes are released which are located on a single aster or in a small truncated spindle simulating the maturation spindle. The mitotic figures remain blocked in this condition until the egg dies. It is interesting that the same reaction may be obtained in mature eggs if these are first made "immature" again by a two-hour exposure to carbon dioxide.

Cytoplasmic Phenomena. The primary polarity of the egg is clearly expressed in the ovarian eggs of most amphibians in the distribution of superficial pigment and of the yolk platelets. From what we know of the origin of the animal-vegetal axis in eggs of invertebrates, it seems likely that it is present in the very young oocyte but initiated, or at least reoriented, by some factor in the ovarian environment. It should be emphasized, however, that the existence of such an external factor has never been demonstrated satisfactorily, contrary to the statements made in some textbooks.

Dorso-ventral polarity, or bilateral symmetry, becomes visible in many amphibian eggs after fertilization, when the grey crescent, which marks the position of the future dorsal lip, appears on one side, at or below the equator. Ancel and Vintemberger (1933) have shown that, in *Rana fusca*, the formation of the crescent is connected with extensive shifts of materials at the egg surface.

In some species, even unfertilized eggs may show bilateral symmetry very clearly. According to Pasteels (1937), certain egg batches of *Rana esculenta* show an oblique pigment boundary, higher on the future dorsal side. The same phenomenon had been described earlier in the axolotl, by Banki (1927, 1929). During the formation of the grey crescent, the whole egg seems to rotate, raising the dorsal side still further. Ancel and Vintemberger (1933) claim that this "rotation" is a purely cortical phenomenon and does not involve the whole mass of the egg. Banki (1929) also applied vital stain marks to the axolotl egg immediately after fertilization. Those on the ventral side remained localized, those on the dorsal side spread in the direction of the median plane during the formation of the grey crescent.

If we are, thus, certain that bilateral symmetry may be present in the egg before fertilization, we also know that the median plane may still be shifted after insemination. In some species, like *Rana fusca*, the position of the point of entrance of the sperm may have an important orienting influence, so that the grey crescent in most cases forms on the opposite side of the egg, as shown most recently by Ancel and Vintemberger (1938d). In other anuran species, there is no constant relation between the point of sperm entry and the median plane (*Rana esculenta*, *Discoglossus*; Pasteels, 1937, 1938). In urodeles, polyspermy would prevent a simple relationship. Even in selected monospermic eggs, the planes of fertilization and of bilateral symmetry do not coincide (*Cryptobranchus*, Smith, 1922; axolotl, Vogt, 1926; Banki, 1927).

The final position of the median plane may also be changed by an artificially imposed rotation of the egg. Ancel and Vintemberger (1938a) placed unfertilized eggs of *Rana fusca* on a slide in an oblique position, with the vegetal pole raised 135°. After fertilization and expulsion of the perivitelline fluid, the eggs could respond to gravity and rotate until they reached the normal equilibrium position. When the grey crescent appeared, it was almost always located on the "descending side" of the egg, *i.e.*, on that side over which the vegetal hemisphere descended during equilibration of the egg. The same result was obtained when the rotation was imposed as late as 60 minutes after fertilization. Beyond this time, it was without effect (Ancel and Vintemberger, 1938b, c). Further experiments showed that the effect of the first rotation may be reversed by a second rotation in the opposite direction, and that the orienting influence of the spermatozoon may be canceled by subsequent rotation (1938e). The observations were extended to *Rana esculenta* and *Triton alpestris* (1938f) and, for *Rana esculenta*, confirmed by Pasteels (1938). There is no doubt that, during the first hour following fertilization, an artificially imposed rotation of the egg can reorient the plane of symmetry. Later on, as the grey crescent begins to form, this treatment is no longer effective. It seems as if an easily displaced substance had now become fixed in a certain area.

Microscopical study of sections through eggs before or after fertilization has long been neglected. Banki (1929) cut axolotl eggs in half along the median plane and saw a broad cortical zone in the animal hemisphere, surrounding a brownish central mass, while the vegetal hemisphere was largely occupied by white, heavy yolk. During formation of the grey crescent, the cortical zone spread out considerably on the dorsal side and formed a thin layer.

More recently, Lehmann (1941, 1945) cut formalin-fixed eggs of the axolotl in two and described a ring of pigmented, sub-cortical or "marginal" plasm in the animal hemisphere which he identifies with the marginal zone of the gastrula, *i.e.*, with the presumptive notochord-mesoderm area. This conclusion is based on the observation that the ring of marginal plasm may be slightly wider on one side. The investigation seems to be of a preliminary nature and should be extended to eggs of various species of amphibians before it can support far-reaching theoretical conclusions.

Undoubtedly, it would be much easier to understand the organization of the egg at fertilization if it could be traced to its origins during the growth period in the ovary which, in amphibians, may last for weeks or months. A highly promising beginning has been made with the aid of histochemical tests, particularly in the hands of J. Brachet (1944). The so-called plasmal reaction, first applied to eggs of axolotls by Voss, indicates the distribution of a special type of phosphatide which is frequently associated with ribose-containing nucleoproteins. In the very young oocyte, the reaction is limited to the "yolk nucleus," a concentration of mitochondria, which later breaks up and forms a peripheral ring. It is interesting that the loci of synthesis of both lipids and proteins coincide with the areas containing plasmalogen.

Ribose-nucleic acid is always present in the nucleoli of the germinal vesicle; in young oocytes, the whole cytoplasm gives an intense reaction; later on, it is largely limited to a perinuclear ring. The germinal vesicle of the full-grown oocyte shows a high concentration of sulfhydryl compounds in the nuclear sap. Following the breakdown of the nuclear membrane, these compounds occupy roughly the same area as the ribose-nucleic acid. These substances spread out from the animal pole, descending, at first, more rapidly on one side, which seems to be the future dorsal side. At the beginning of gastrulation, they are concentrated particularly at the animal pole and in the dorsal lip of the blastopore. It is very tempting to connect the peculiar distribution of ribose-nucleic acid compounds with the origin of bilateral symmetry, although Brachet himself strongly emphasizes the hypothetical character of such a connection.

Experimental Tests of Invisible Organization of Egg Cytoplasm

Maps of Organ-Forming Territories. Although we are well informed of the prospective significance of the various regions of the blastula and gastrula, corresponding maps of the unsegmented egg have not been published so far, perhaps because of technical difficulties involved in applying vital stain marks at sufficiently early stages, before the cortical shifts described by Ancel and Vintemberger begin. Such maps would be extremely valuable in the analysis of the rearrangements of materials that take place after fertilization and during early cleavage, as is clearly shown by Banki's observations and by the studies on ingression which are discussed in detail in the paper by Dr. Nicholas.

Effects of Gravity and Centrifuging. The effects of gravity on amphibian eggs have been studied repeatedly since the days of Pflüger, Schultze, and Born, who turned frog's eggs upside down and forced them to develop in this inverted position, either by compressing them between two slides, or by preventing the swelling of the jelly and the formation of the normal perivitelline space. The resulting embryos were abnormal, largely because of disturbances of gastrulation which often began at two different points. The observations have always been rather difficult to interpret because we do not know in sufficient detail how the various substances within the egg are redistributed under the action of gravity. Pasteels (1941), who recently inverted eggs at the time of fertilization, also seems to have limited his observations largely to living eggs. On that basis, he constructed optical sections which indicate that the variable results may be explained by varying degrees of descent of the heavy yolk. In most cases, two blastopores are formed; no normal embryos appear, probably because gravity alone is unable, in the time available, to assure a complete reversal of the original orientation.

However, if inverted eggs are centrifuged at 460 gravities for from one to five minutes, a complete reversal of the original animal-vegetal (= antero-posterior) axis may take place. Gastrulation is unitary, and about one-half of the resulting embryos are perfectly normal, although their cephalic end corresponds to the original vegetal pole of the egg. The complete reversal of the primary polarity of the egg by mild centrifuging makes it appear unlikely that polarity is determined primarily by properties of the egg surface, since the cortex does not seem to be greatly affected by inversion or centrifuging.

Pasteels (1940b) also centrifuged eggs of *Rana fusca* which were free to rotate and to orient themselves with the animal pole pointing centripetally. Following centrifugation of two to three minutes, at 460 gravities, gastrulation was often normal. In spite of this fact, the axial organs were sometimes completely absent in all or part of the embryo. In some cases, the tail still contained an axial mass of recognizable somites while the trunk was completely unorganized beyond the formation of a mantle of mesoderm. Other embryos were completely anaxial although they differentiated numerous blood cells.

If eggs are centrifuged after formation of the grey crescent, fewer embryos survive, but the resulting tadpoles may be completely normal. The formation of the axial organs, or organogenesis in general, is no longer inhibited. From these observations Pasteels concludes that, at the time of fertilization, the egg contains a substance of relatively low specific gravity which is a precursor of the active principle of the organization center, called organisin. Perhaps this precursor is identical with the ribose-nucleic acid compounds which Brachet found in higher concentration on the dorsal side of the egg. Centrifuged eggs actually show these compounds concentrated at the animal pole. Once the grey crescent has been formed, the precursor becomes fixed in a more cortical area and cannot be displaced by centrifuging.

Pasteels (1940a), repeating still another old experiment, rotated eggs, following the formation of the grey crescent, through 135° and forced them to develop in this position by compression between two slides. The rearrangement of the materials is more uniform than after complete inversion, since the heavy yolk tends to sink down along one side of the egg. However, in its new position, it will have various spatial relations to the old grey crescent area. While the blastopore always appears at the boundary of the heavy yolk mass, its position is also influenced by the original location of the grey crescent. Very often, the point at which invagination begins seems to represent a compromise between these two forces.

These experiments form the basis of a theory of development (Dalcq and Pasteels, 1937, 1938; Dalcq, 1938) which recognizes two important features of the egg at fertilization: an animal-vegetative vitelline gradient, involving the whole mass of the egg, and a dorsal field limited to the cortex. As cleavage proceeds, an interaction between these two components takes place which is visualized as a simple chemical reaction producing different results at different points, because of the varying concentration of the vitelline substance "V" and the cortical substance "C." The products of this reaction would then create a field of "morphogenetic potentials" which, in turn, would determine the fate of various regions of the embryo.

This theory, which in its details is somewhat more complex than the brief résumé indicates, has the great merit of focusing our attention on the yolk material which is either not as inert as was formerly believed, or always associated with some other, active material. Also, it seems highly probable that interactions between the vitelline gradient and a more superficial dorsal "field" play an important role in the earliest stages of development. The theory, which undoubtedly will be subjected to more experimental tests, has been criticized by Rotmann (1943) and Lehmann (1945). The latter questions the existence of a true yolk gradient in the amphibian egg and claims that distinct masses of heavier and lighter yolk are present. In his opinion, the dorsal field is not cortical, but sub-cortical or "marginal." It is very likely that continued investigations will bring about a *rapprochement* of these conflicting views.

Isolation of Parts of Egg. The invisible organization of the cytoplasm of the egg before and during cleavage may also be studied by isolating parts of the egg, to test their developmental potencies. The interpretation of the results obtained by such methods is somewhat simpler since it may be formulated in terms of the organizer concept.

Spemann (1902) showed that eggs of *Triton* in early gastrula stages may be constricted within the jelly, by means of a loop of fine hair. When the loop divides the blastopore symmetrically, *i.e.*, when the plane of constriction coincides with the median plane of the embryo, complete twins are formed. When the gastrula is constricted in the frontal plane, so that the dorsal and ventral halves are isolated, the dorsal embryo alone develops normally; the ventral one may gastrulate but fails to form any axial organs (somites, notochord, and neural tube).

Similar results are obtained by dividing the blastula or earlier cleavage stages, or by isolating the two blastomeres at the two-cell stage (Spemann, 1901; Spemann and Falkenberg, 1919; Ruud and Spemann, 1922; Ruud, 1925). It is, thus, necessary to conclude that the dorsal and ventral sides of the egg are already different from one another at this early stage. Spemann was inclined to believe that the future center of organization becomes localized on the dorsal side of the egg before the first cleavage, perhaps at the same time as the grey crescent forms in eggs of other species of amphibians, *i.e.*, about one to two hours after fertilization.

However, if eggs of *Triton palmatus* are constricted as early as twenty minutes after fertilization, we still obtain the same results: about one-fifth of the fragments containing the diploid cleavage nucleus give rise to typical ventral embryos (Fankhauser, 1930b), a similar proportion as that produced in experiments on the two-cell stage. The ventral embryos may survive for several days and form mesenchyme, blood cells and pigment cells, but no trace of notochord, myotomes, or neural tissue ever appears (FIGURE 4). This demonstrates that an important difference

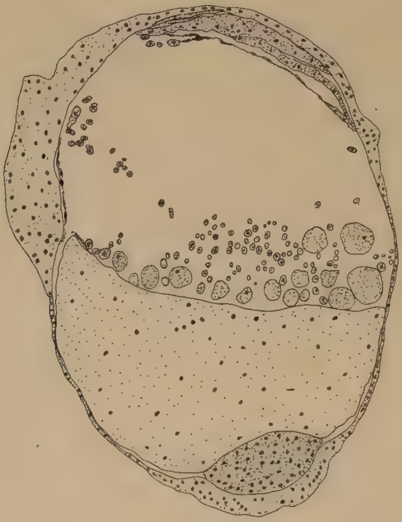

FIGURE 4. Section, along animal-vegetal axis, through typical ventral embryo, developed from a diploid egg fragment of *Triton palmatus* 6 days old (control embryo shows well differentiated neural tube, gill buds, and indication of forelimb buds). The egg was constricted twenty minutes after insemination. Note presence of mesenchyme near animal pole, blood island (a typically "ventral" differentiation) near vegetal pole, irregular thickening of ectoderm, complete absence of axial organs.

in developmental potencies exists between the dorsal and ventral sides of the egg within a few minutes from fertilization. The formation of the grey crescent seems to be a secondary phenomenon which makes this difference clearly visible in the eggs of some species. It is possible that the basic dorso-ventral differentiation is present before fertilization. However, attempts to obtain egg fragments by constriction of unfertilized eggs in the posterior part of the oviducts have not been successful so far.

There is no direct evidence that the dorsal area of the unsegmented egg already has all the properties of the center of organization of the blastula or gastrula, *e.g.*, that, on transplantation, it could at once induce formation of a secondary embryonic axis. Inductions have been obtained by bringing unsegmented eggs or $\frac{1}{4}$ blastomeres in contact with ectoderm of young gastrulae for 30 to 35 hours (Mayer, 1939). However, during this interval, the eggs developed at least to the blastula stage so that it is not possible to ascertain the exact time at which inductive powers arise. As far as the analysis of the cytoplasmic organization is concerned, the fact that the dorsal half of the egg has the capacity to form an organization center while the ventral half does not, is in itself important enough.

Experiments on the two-cell stage have the advantage that both cells develop with identical, diploid nuclei and may serve as mutual controls. If the two halves of the unsegmented egg are separated completely, both may develop because of the presence of supernumerary sperm nuclei. In rare cases, following constriction in the median plane, one may obtain twin larvae, one diploid, the other haploid. However, for our purposes, it would be advantageous to supply both halves with identical, diploid nuclei. Spemann (1914, 1928) showed that this may be done by

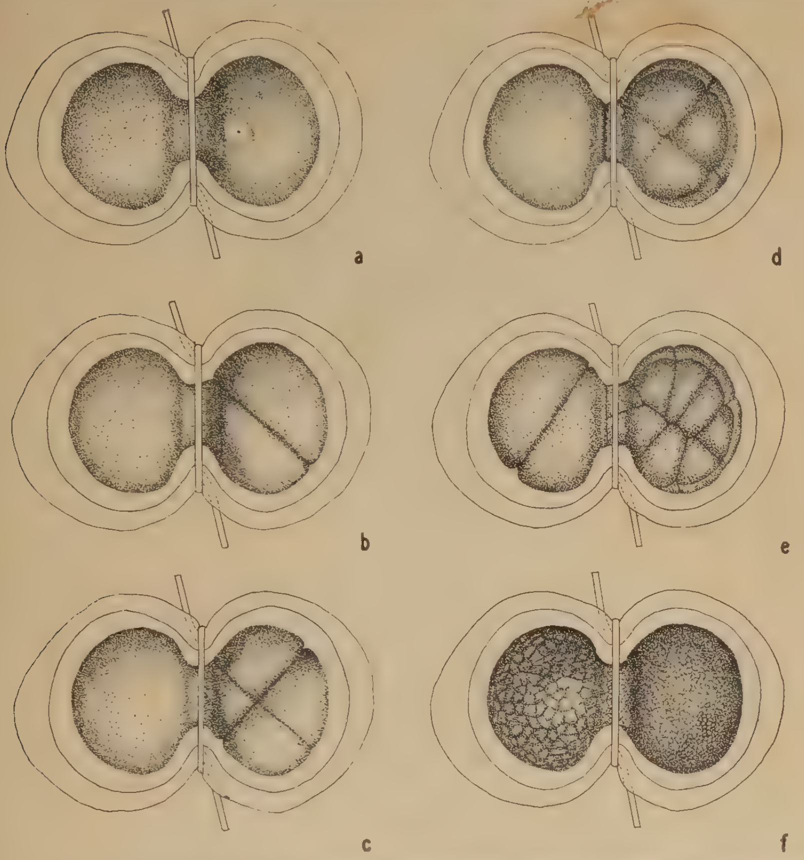

FIGURE 5. Drawings of cleavage of a partially constricted egg of *Triturus viridescens*, illustrating "delayed nucleation" of left-hand part. Magnification about 14 times.

a, immediately after constriction (about 20 minutes after insemination). Small black spot in center of light area, to right of hairloop, indicates position of second maturation spindle. Larger dark spot above this area marks point of penetration of a spermatozoon.

b, first cleavage of right half containing egg and sperm nuclei.

c, second cleavage; one of the four cells connects with bridge, allowing its nucleus to enter.

d, third cleavage; the nucleus in the bridge has divided and caused formation of a cleavage furrow to the left of center of bridge. One of the daughter nuclei (*i.e.*, one of the eight nuclei of the eight-cell stage) has entered the left half.

e, fourth cleavage; first division of left half.

f, blastula stage; delay in beginning of cleavage of left half is clearly indicated by larger size of the cells.

partial constriction of the egg which leads to "delayed nucleation" of the originally non-nucleated half. The development of supernumerary sperm nuclei is usually inhibited in both halves of a partially constricted egg, as long as the connecting bridge remains wide enough (Fankhauser, 1925, 1930b).

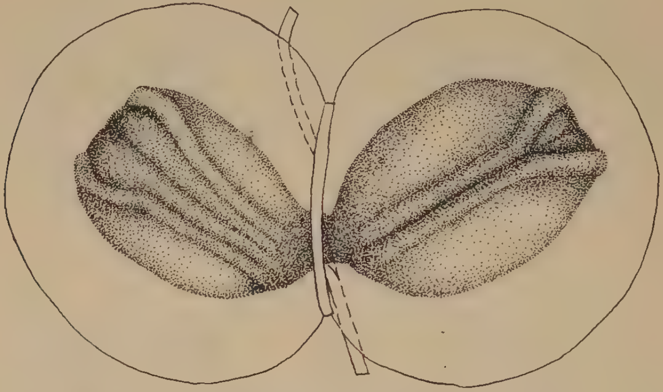

FIGURE 6. Neurula stage of egg that was partially constricted in future median plane, shortly after fertilization. Normal twin embryos. Difference in time of beginning of cleavage of the two halves is still clearly shown. Magnification about 25 times.

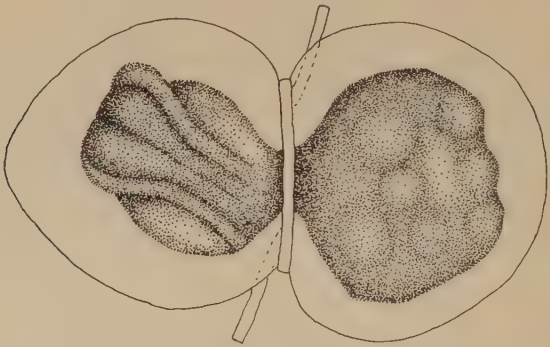

FIGURE 7. Neurula stage of another egg that was constricted in future frontal plane. Dorsal half develops normally, ventral half unable to form axial organs (right).

The experiment has been repeated many times with eggs of different species, *e.g.*, *Triturus viridescens* (FIGURE 5; *cf.* Fankhauser, 1932b). Cleavage begins in the half containing the fusion nucleus. Sooner or later, depending on the diameter of the connecting bridge, a descendant of the original nucleus will enter the bridge and divide there, as may be seen from the formation of a cleavage furrow. One of the daughter nuclei moves into the center of the uncleaved half and initiates a delayed cleavage which proceeds normally. As in experiments on the two-cell stage, one may obtain normal twins (FIGURE 6), or one normal and one ventral embryo (FIGURE 7). If the connection between the two parts of the egg is maintained during gastrulation, a double-headed monster may result

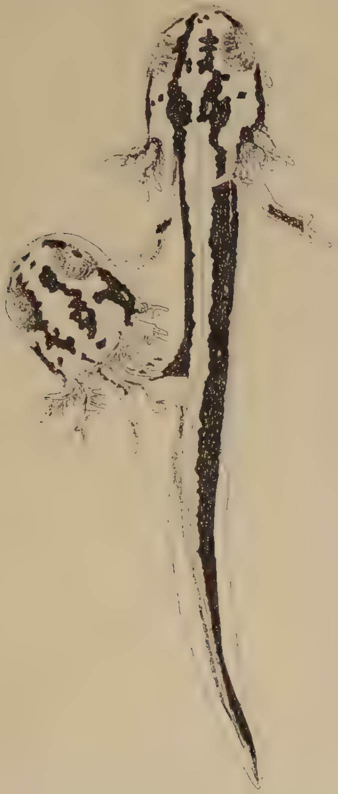

FIGURE 8. Two-headed larva developed from partially constricted egg, shown in neurula stage in FIGURE 6. 39 days old. Magnification about 14 times.

(FIGURE 8). If the bridge breaks, or is severed artificially by tightening of the hairloop, two normal, though slightly asymmetrical larvae may be produced (FIGURE 9).

Furthermore, following both complete and incomplete constriction of unsegmented eggs, a whole series of intergrades between ventral and normal embryos makes its appearance: embryos with weak axial organs, larvae with microcephaly of various degrees (FIGURES 10 and 11), larvae with strong unilateral defects limited to the head or trunk region (Fankhauser, 1930b, 1932b; Streett, 1940). This may be explained by the observation that the plane of constriction may form any angle with the invisible median plane of the egg so that the future center of organization is distributed between the two halves in various proportions. If eggs are constricted very lightly following fertilization, so that they are immobilized within the jelly capsule, the relation between the plane of constriction and the plane of symmetry may be determined at the time of appearance of the blastopore. Twenty-two eggs of *Triturus viridescens* tested in this way showed the following positions of the hairloop:

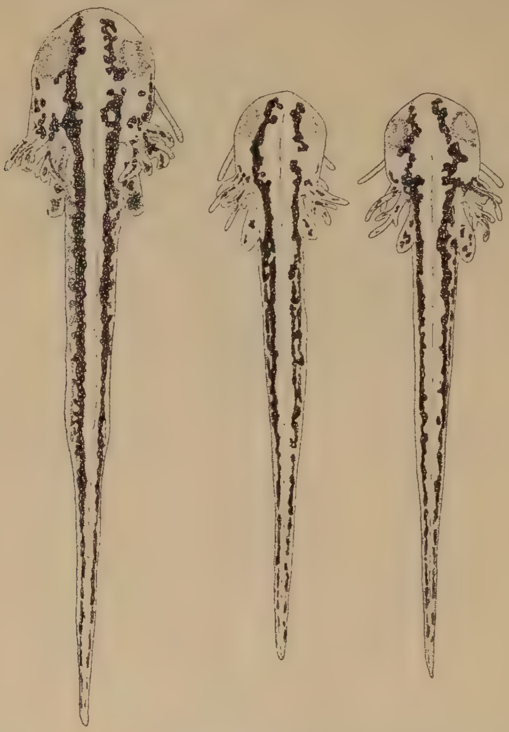

FIGURE 9. Control larva and twins produced by partial constriction of unsegmented egg in median plane. The connection between the two halves of the egg broke after gastrulation. Note smaller size of eye, balancer, and forelimb bud on right side of left-hand twin, smaller size of left forelimb bud of right-hand twin.

in median plane	3
small angle with median plane	4
oblique	8
small angle with frontal plane	3
in frontal plane	4

On the basis of the results of experiments on the early gastrula stage, where the distribution of the dorsal lip area between the two halves may be observed directly, an egg fragment with a small lateral portion of the organization center would be expected to develop into a micro-axial or microcephalic embryo, while a fragment obtaining approximately one-half of the center would give rise to a "left" or a "right" larva with slight unilateral defects. The similarity of the types of abnormal embryos produced by halves of the unsegmented egg and of the gastrula raises the question to what extent regional differences in the future center of organization may be present at fertilization.

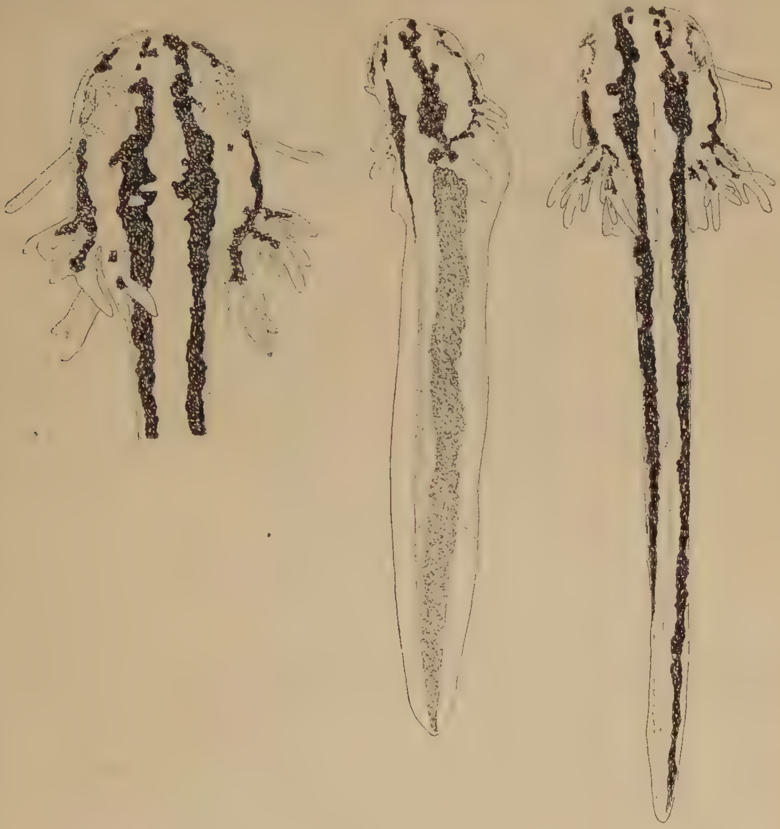

FIGURE 10. Control larva and twins developed from an egg that was constricted in an oblique plane, distributing area of future organization center unequally between the two halves. One twin normal, the other microcephalic and more retarded in development.

One more abnormality must be mentioned. Following median or nearly median constriction of the egg, the "left" twin, characterized by various degrees of underdevelopment of its right side, always has the heart and viscera in their normal position. Among the "right" twins, about 50 per cent show complete *situs inversus*. Reversal of asymmetry occurs whether the division of the egg takes place in cleavage or blastula stages (Spemann and Falkenberg, 1919), or shortly after fertilization (Fankhauser, 1930b). This indicates strongly that the typical asymmetry of the vertebrate body may be traced back to some slight difference in the invisible organization of the right and left halves of the egg at fertilization.

In the early days of experimental embryology, it was customary to make a sharp distinction between the eggs of Anurans and those of Urodeles. The former were supposed to show more "mosaic" organization, the latter to be examples of the "regulative" type. Although there undoubtedly exist differences in the degree of self-differentiation and of regulation between eggs of even closely related species of amphibians, there is no such clear-cut division between those of the two orders. Once the technical difficulties were overcome, Schmidt (1933), working in Spemann's laboratory, could show that constriction of eggs of *Bombinator* and of *Rana* produces essentially the same results as that of newts' eggs.

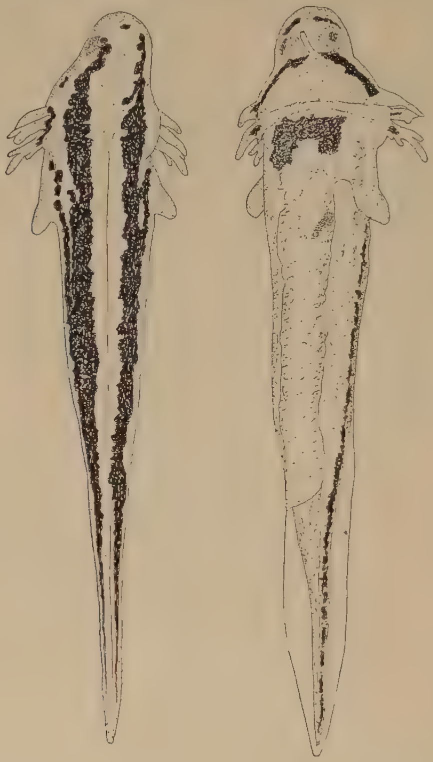

FIGURE 11. Dorsal and ventral views of a highly microcephalic larva developed from an egg fragment. The twin embryo was lost. Single balancer in ventral midline, between eyes.

While differences along the dorso-ventral axis are well established, those along the animal-vegetal axis have hardly been investigated. We need more information on the potencies of the isolated micro- and macromeres of the eight-cell stage, and of animal and vegetal fragments of unsegmented eggs and of cleavage stages, in extension of the work of Votquenne (1933), Vintemberger (1934, 1936), Streett (1940), and Stableford (1939). Such experiments are particularly important since tests of the blastula and gastrula stages have demonstrated a considerable capacity for self-differentiation on the part of the presumptive endoderm. Furthermore, there is the possibility of an early segregation of some material essential for the formation of the germ cells which, according to Bounoure (1934), takes place near the vegetal pole of the frog's egg before cleavage begins.

Analysis of the Nucleus

A discussion of the organization of the amphibian egg at the beginning of development would be very incomplete without a consideration of the nucleus and chromosomes. In recent years, geneticists have become increasingly interested in the cytoplasm of the egg, for several reasons. First, it is in the cytoplasm that most of the genes produce their effects. Second, presence of certain essential substances in the cytoplasmic substrate in limited amounts may explain some special phenomena, like competition between genes. Finally, the recent work of Sonneborn (1943) and Spiegelmann (1946) and others calls for the presence, in the cytoplasm, of self-reproducing units of nucleo-protein nature, probably derived from genes, which control the synthesis of proteins and enzymes. It is obvious that the embryologist, in turn, can contribute to the synthesis by paying more attention to the possibilities of experimenting with the chromosomes of the egg. I should like to mention briefly some lines of approach that may be followed:

a. Spemann's experiments on partial constriction demonstrated that a single nucleus of the eight- or sixteen-cell stage, which migrates into the non-nucleated half, is sufficient to initiate normal development of that half. Would a single nucleus of a more advanced stage, perhaps of an embryo, still be able to take the place of the normal fusion nucleus? Rostand (1943), repeating an experiment of Bogucki's, smeared unfertilized eggs of *Rana fusca* with embryo pulp obtained from a gastrula, then punctured them with a needle to induce parthenogenesis. Unsmeared control eggs gave the usual small number of dwarfed, unviable, haploid larvae. Smeared eggs produced similar tadpoles, but also a number of more vigorous larvae of normal size which survived for three to six weeks and were possibly diploid. Rostand suggests that, in these cases, a nucleus from one of the gastrula cells on the surface was dragged into the egg by the stylet and took over the functions of the cleavage nucleus, replacing the egg nucleus. It would be interesting to repeat the experiment on a larger scale and to work out the cytological details.

b. We know from observations on androgenetic salamander eggs and egg fragments that cell division may proceed in the complete absence of chromosomes. A blastula may be formed which includes large areas of non-nucleated cells containing actively dividing asters. Such eggs do not survive gastrulation (Fankhauser, 1934a; Fankhauser and Moore, 1941b). Thus, we know that the presence of chromosomes is necessary for normal development from this stage on. Other observations show that the requirements of the egg are more precise. From the time of gastrulation on, it needs at least one complete, balanced set of chromosomes. In the absence of the egg nucleus, sperm nuclei frequently form multipolar mitotic figures which result in abnormal distribution of chromosomes. With such varied, unbalanced sets of chromosomes present in different cells, development again comes to a standstill at the gastrula stage (Fankhauser, 1932b, 1934b; Kaylor, 1941).

On the other hand, complete sets of chromosomes may be subtracted or added with relatively slight effect on early development. Essentially normal development to early larval stages may take place in newts and other salamanders with anywhere from one to five sets of chromosomes, in spite of the fact that nuclear and cell size change in approximate proportion to the chromosome number (review in Fankhauser, 1945). Within a wide range of cell sizes, organogenesis is independent of the size of the individual building units.

c. The role of the chromosomes in development may also be studied by suitable crosses between various species of amphibians, some of which lead to an early arrest of development. As an example, we may mention the results of the genus cross between *Triton palmatus* and *Salamandra maculosa*, studied in Baltzer's laboratory by Schönmann (1938) and Lüthi (1938). Development invariably stops at the gastrula stage, when cells begin to die in various regions until the whole egg breaks up, although some cells still appear normal at this time. Signs of a lethal effect may be detected in the early blastula, when chromosomes frequently lag at anaphase and telophase. Obviously, there is some incompatibility between the *Salamandra* chromosomes and their *palmatus* environment which makes further development impossible. However, if a healthy piece of a hybrid gastrula is transplanted to a normal *Triton* gastrula, it may survive and form a harmonious part of various organs, such as the hindbrain and ear, with all the associated cartilages and muscles. Although other interpretations are possible, it seems likely that the cells of the transplant, although still normal at the time of the transfer to the normal host, were destined to die soon because of their hybrid constitution. Following transplantation, some factors residing in the healthy tissues of the host enabled the cells of the graft to overcome their inherent deficiency. Such stimulating effects of the adjacent normal host tissues have been demonstrated conclusively by Hadorn (1935, 1937) in chimaeras composed of parts of unviable merogonic hybrids and of normal embryos.

d. Time does not permit us to discuss in detail the experiments on hybrid androgenesis in which the maternal chromosomes are eliminated following cross-fertilization between two species. One should mention, at least, Hadorn's (1936) now classical experiment because it has a bearing on our interpretation of the cytoplasmic organization of the egg. In an embryo the cells of which contain cytoplasm of *Triton palmatus* and haploid nuclei of *Triton cristatus*, an incompatibility manifests itself soon after the closure of the neural folds, primarily in the head mesoderm. All embryos die at this stage (Baltzer, 1930). If a large piece of presumptive epidermis is taken from a still healthy embryo in the gastrula stage and transplanted to a normal gastrula of a third species, *Triton alpestris*, it will survive for months, until the host completes metamorphosis. At that time, specific characteristics appear in the epidermis. In *Triton cristatus*, the surface is smooth, while in *Triton palmatus* it shows toothlike projections formed by rows of flattened cells. The hybrid skin, with chromosomes of *Triton cristatus* and cytoplasm of *Triton palmatus*, shows the structure of the maternal species. This indicates that this characteristic was determined very early, while the egg was still in the ovary, under the influence of the maternal gene complex. The cytoplasm of the egg at fertilization would then contain some element which is responsible for the development of the characteristic skin pattern several months later. The recent work of Porter (1941) and of Moore (1946) on reciprocal hybridization and hybrid androgenesis between different local forms of *Rana pipiens* shows that other characteristics, appearing earlier in development, may also be influenced by the constitution of the cytoplasm of the egg.

Conclusions

The organization of the amphibian egg at fertilization, as it is known today, includes at least the following features:

(*1*) There must be a basis for the primary animal-vegetal polarity which may be connected, at least in part, with the distribution of yolk and associated substances rather than the properties of the egg surface alone, since the direction of animal-vegetal polarity may be completely inverted by centrifuging.

(*2*) There must also be a material basis for the bilateral symmetry of the embryo, *i.e.*, the dorso-ventral polarity, which is certainly present within a few minutes after fertilization, and may exist before. The direction of this axis may be shifted by secondary factors, such as a rotation imposed on the egg before the end of the first hour or, in some species, by the point at which the sperm enters the egg. There are indications that the dorso-ventral organization may be connected with the early localization of sulfhydryl and ribose-nucleic acid compounds derived from the germinal vesicle or from the cytoplasm surrounding it. These compounds, in turn, may be essential for the functioning of the future organization center, and for organogenesis in general.

(*3*) The typical asymmetry of the vertebrate body, which is expressed later in the position of the heart and viscera, also seems to be foreshadowed in the organization of the ooplasm. The nature of this factor is entirely unknown.

(*4*) The structure of the egg may be still more complex because of the presence of regional differences in the future organization center or in the presumptive endoderm.

(*5*) There are many indications of early interactions between cytoplasm and nucleus, for instance, the inhibition of the supernumerary sperm nuclei in the urodele egg, the normal coordination between chromosomal and centrosomal cycles of mitosis, and the peculiar reaction of the spermatozoa in immature eggs. All these adjustments are essential for the realization of normal cleavage.

(*6*) Slightly later, at the time of gastrulation, interactions between genes and cytoplasm assume importance, as is shown by the results of abnormal distribution of chromosomes through multipolar mitosis and by many hybridization experiments.

(*7*) Observations on reciprocal hybrids and on hybrid androgenesis show that various characteristics, appearing early or late in development, may be influenced by the constitution of the cytoplasm of the egg. The organization of the egg at fertilization may thus be complicated by the presence of components that are responsible for the appearance of such maternal characteristics.

(*8*) It is doubtful that we shall reach a real understanding of the already complex organization of the egg at fertilization unless we make a determined effort to trace its origins back into pre-fertilization stages, to the growing oöcyte in the ovary. During the weeks or months which the oöcyte passes in its follicle, the foundations of the future individual are laid down while all visible signs point to intense metabolic activity. Even the chromosomes take time out from their preoccupation with meiosis and undergo the profound and peculiar changes resulting in the still poorly understood "lampbrush" appearance, because they also are taking part in the synthesis of the elements of a new individual that will resemble the old. The young germ should be in an ideal condition to respond to some of the old questions of the embryologist. To be sure, it is less accessible to experimental procedures than it will be later on, but it may not prove to be as unapproachable as it is commonly supposed to be.

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Discussion of the Paper

Dr. Robert Chambers (*New York University, New York, N. Y.*):

I wish to continue the point I raised in the discussion of Dr. Costello's paper in which I suggested that the sperm or monaster in many marine eggs may be concerned with the reorientation of formative stuffs. The monaster in the larger and more highly lecithal amphibian egg does not develop to the extent that it does in the echinoderm egg in which the entire egg substance is involved. Is it possible that the lack of a sufficiently extensive aster permits dislocation in these large eggs by fragmentation, or by abnormal sedimentation, or centrifugation to upset the organization pattern?

Dr. L. T. Stableford (*Department of Biology, Lafayette College, Easton, Pennsylvania*):

There is evidence from the cytological observations of Schultze, Hibbard, and others that the polarity of the amphibian egg is determined at an early stage of the oöcyte since vitellogenesis is initiated excentrically in that portion of the oöcyte which later becomes the vegetal hemisphere of the egg. With this in mind, I would suggest that the relation between yolk and cytoplasm in the egg is more than "gravitational," that it is, rather, intimately structural.

This concept of a yolk-cytoplasm complex in the vegetal hemisphere runs contrary to the conclusions commonly drawn from inversion and centrifugation studies. It is usually assumed that, when the egg is inverted, the yolk spherules change position under the influence of gravity and mechanically force a reorganization of the egg. It should be pointed out, however, that this experiment involves, in addition to gravity, the effect of mechanical pressure since the egg must be compressed between glass plates or in a tube to invert it. Granting that Pflüger showed pressure itself to be without effect on the development of the egg, it seems reasonable to suggest that the combination of pressure and gravity disturbs the structure of the egg so radically that the shifted material is actually a yolk-cytoplasm complex.

Further evidence for a yolk-cytoplasm complex as a structural component of the egg comes from the work of Todd in 1940. He indicated that, when the amphibian egg is centrifuged, there is first a movement of liquid material to the animal pole of the egg and that this material flows between the large yolk spherules; it is apparently only when the centrifugal force is strong that the characteristic packing of yolk at the vegetal pole occurs.

I bring up this idea of a possible yolk-cytoplasm complex mainly to call attention to the internal portions of the egg since interest seems to be centering almost exclusively on the cortex or surface coat at present.

Dr. G. Fankhauser:

In reply to Dr. Chambers, let me say that observations on eggs of several species of amphibians have shown that the fully developed sperm aster is very large and may extend through the entire animal hemisphere. However, the maximum size is not reached until two and a half hours after fertilization (in eggs of newts). While the growth of the sperm aster thus may be an important factor in the rearrangement of certain egg materials, there is no direct evidence that it is instrumental in the segregation of developmental potencies in different parts of the egg.

The views expressed by Dr. Stableford are in general agreement with those developed by Dalcq and Pasteels, who emphasize the active role of the "yolk" in early development. Future investigations of this problem should include a more exact determination of the roles played by the material of the yolk platelets themselves and by other substances that might be closely associated with these bodies.

SIGNIFICANCE OF THE CELL MEMBRANE IN EMBRYONIC PROCESSES

By JOHANNES HOLTFRETER*

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EVIDENCE of the embryological significance of the cell membrane may be derived from a consideration of this structure in connection with the following phenomena: cellular permeability, amoeboid motility, morphogenetic movements, cytoplasmic division, selective adhesiveness, cell polarity, and cellular differentiation. It is clear that such a wide range of phenomena cannot be discussed exhaustively within the frame of the present review. The aim of this paper is, therefore, to give not more than a tentative synopsis of the embryological functions of the cell membrane and to discuss the physico-chemical and physiological properties of this structure only as they may serve this purpose. This paper will mainly be concerned with observations on amphibian development, although it is realized that some of the phenomena to be touched upon have been more thoroughly investigated in other forms, such as the echinoderms. The discussion will include numerous unpublished observations.

Functions of the Coat in the Amphibian Egg

Physico-Chemical Properties of the Coat. The array of protective envelopes investing a fertilized amphibian egg resembles that described for the sea urchin egg by Chambers (1938, 1940). Apart from the external gelatinous layers and the vitelline membrane, the amphibian egg and its subsequent stages are covered by a film, or coat, which is firmly attached to the underlying cell membrane and which seems to be comparable to the hyaline layer in echinoderm eggs. The integrity of the coat requires the presence of small amounts of calcium in the immersion fluid (Holtfreter, 1943a). Amphibian embryos which are placed in an isotonic solution lacking calcium ions fall apart into single cells, and a mucilaginous substance passes into solution. Similar mucus formation and disaggregation occur when the pH of the balanced salt solution is raised above 9.6 or lowered below 4.2, or when solutions of sodium citrate or oxalate are applied. As in the case of disintegrated echinoderm blastomeres, the amphibian cells reaggregate and survive the treatment if, within a restricted period, calcium is restored to the external medium. A new coating substance may be secreted by the outermost surface of the ectodermal and endodermal cells.

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* In dedicating this paper to Professor K. von Frisch, I am expressing my great admiration for his scientific work, and my indebtedness to him for the inspiration and kind assistance I received while I was a member of his former Zoological Institute at Munich.

In sea urchin eggs, the hyaline coat evidently consists of proteins. Excess of calcium in the sea water renders this layer rigid and insoluble, while acidulated sea water (pH 3.5) makes it disappear (Moore, 1928). The hyaline layer is furthermore attacked by trypsin, and it is negatively birefringent in the radial direction, indicating the presence of tangentially oriented protein micells (Runnström, Monné, and Broman, 1943). Based upon the data on the susceptibility of the coat in amphibian embryos to acids, bases, and calcium-free solutions, it may be assumed that here, too, proteins constitute the essential components of this structure. Alcohol and other protein coagulants render the coat brittle.

Dissection experiments on living amphibian embryos show that the coat is apparently not a living and indispensable part of the egg and of the epithelia deriving from the egg surface. While covering and interconnecting the peripheral cells as a syncytial layer, the coat, where it forms intercellular bridges, can be drawn out between glass needles into long contractile threads which may be cut off without affecting in any way the viability of the attached cell. The surface of the coat is normally semi-solid and non-adhesive, capable of forming wrinkles when stretched, and being in the living egg in a state of elastic tension. Because of these properties, the coat represents the essential structure which unites the blastomeres into a closely packed body.

As compared with the proximal, uncoated side of the surface epithelia, the coated cell surface is considerably less permeable to water, electrolytes, and various toxic substances. Whereas whole embryos, or embryonic fragments, which are entirely covered by a layer of coated cells can be cultured for long periods in strongly hypotonic media containing traces of calcium, uncoated fragments cytolyze in it within a short time. Uncoated cells also show an enhanced susceptibility to hypertonic solutions and vital dyes. It is this reduced permeability of the surface layer which enables the amphibian egg to develop normally in tap water.

A protective coat does not seem to exist in the ovarian eggs of the frog, since isolated eggs of this stage die rapidly in various solutions which do not impair the viability of mature eggs. On the other hand, overripeness is associated with a softening of the egg surface, which is reflected in its reduced resistance to centrifugation and its reduced capacity of closing wounds. It is not unlikely that the abnormal cleavage and gastrulation patterns observed in fertilized overripe frog's eggs (Witschi, 1930; Briggs, 1941) are predominantly due to a partial disintegration of the egg surface, involving probably both the coat and the underlying plasma membrane.

Susceptibility to Mechanical Agitation. This double layer disintegrates readily when disturbed mechanically. The susceptibility can be demonstrated by placing a number of fertilized frog's eggs, with all their coverings intact, in a bag of cheesecloth and letting a quick succession of water drops hit the external jelly of the eggs. If the drops fall from a height of not more than about 10 cm., the eggs suddenly swell strongly and disintegrate within an hour. It is surprising that the eggs succumb to the rhythmic percussions while they are still enclosed in their protective envelopes and without being visibly deformed by the impact of the falling drops. The observation recalls the early experiments of Dareste (1891) which have recently been taken up by Landauer and Baumann (1943), and which showed that shaking of unincubated chicken eggs tends to produce drastic developmental abnormalities. Fauré-Fremiet (1932) and Battle (1948) found that the eggs of various fishes become rapidly liquefied or develop abnormally when mechanically agitated. Similar liquefying effects of shaking or other mechanical irritations have been observed in Protozoa, bacteria, erythrocytes and other cells (Koelsch, 1902; Angerer, 1936; Chambers, 1924). Frequently, death of the cells is preceded by the formation of hyaline blisters and by considerable swelling, which suggests that disintegration is initiated by a molecular disarrangement of the surface membrane, producing at first increased permeability, and subsequently breakdown (see p. 731).

Significance of the Coat in the Process of Gastrulation. When the cells of an amphibian gastrula enter into the phase of morphogenetic movements, it is in the first place the investing coat which integrates the amoeboid activity of the individual cells into the coordinated and synchronized movements of entire germ layers (Holtfreter, 1943b, 1944). By virtue of this superficial elastic sheet which cements the peripheral cells together into a continuous layer, gliding movements of the cells in any region of the gastrula are transmitted to adjacent regions. Similar effects of a tangential pull can be observed in a layer of ectoderm which is proceeding to close in on a wound inflicted to this layer. It should be emphasized, however, that the motive forces for the gastrulation movements of spreading and invagination cannot be ascribed to the properties of the coat, but originate within the living cells proper. Embryonic cells which are not held together by a syncytial coat are perfectly capable of spreading over an organic or inorganic substratum, and they will slip, singly or in groups, into the depth of a layer of endoderm with which they have been brought into contact. The coat merely regiments these amoeboid movements so that they become collective events. Actually, the tensile strength and contractility of this structure counteract the movements of invagination. Cells which migrate singly into the interior of a gastrula can do so only by detaching themselves from the coat. Where such a detachment does not occur, the invaginating cells become stretched into cylindrical or even filiform bodies, which, through their combined efforts, draw the coated surface inward in the form of an archenteron or other cavities.

It follows from the above considerations that agents which reduce the tensile strength of the coat, or remove the layer altogether, will have drastic effects on the morphogenetic movements, although invagination will not necessarily be suppressed. Agents having such a dispersive or liquefying effect on the coat have already been mentioned; they are salt solutions which are hypertonic, or free of calcium, or have a pH above 9.6 or below 4.4. The suppressive effect of lithium on the morphogenetic movements in amphibian embryos does not seem to differ markedly from that of other monovalent cations.

A weakening of the coat results in exogastrulation. The movements most inhibited are the epiboly of the ectoderm and the involution of the endoderm, whereas the mesodermal tissues freely invaginate into the interior of the endoderm (Holtfreter, 1933). Ectoderm, when its coat has been removed or has lost its original non-adhesiveness, may even become embedded within the endoderm. Morgan (1903), by applying hypertonic solutions of lithium chloride, obtained such a partial inversion of the germ layers in frog embryos. In a similar way, by making use of the coat-dissolving action of sodium citrate (hypo- to hypertonic solutions), the writer was able to produce frog gastrulae in which the entire "decoated" ectoderm, together with the mesoderm, sank into the interior, while the endoderm became the external layer of the whole embryo.

Exogastrulation can be brought about either by culturing pre-gastrula stages permanently in solutions having only a softening effect upon the coat, or by subjecting the early gastrula for a brief period to media, such as alkali or sodium citrate, which produce an immediate and total breakdown of the coat, then returning them to physiologically normal conditions. The idea of Jenkinson (1906) that hypertonicity of the external medium is not the sole agent which causes exogastrulation, is strongly supported by these experiments where the agents were applied in hypotonic solutions, and where the treatment lasted not longer than 15 to 20 minutes. This gave the disaggregated cells full opportunity to reestablish the continuity of the embryo and to continue differentiating.

It would be unwise to attempt an explanation of the whole exogastrulation syndrome in terms of a weakening or dispersal of the coating substance. However, it is reasonable to assume that the integrity of the coat is at least one of the most important factors safeguarding the normal display of the movements of gastrulation and neurulation.

Fate of the Coat in Later Stages of Development. In consequence of the infolding of all except the prospective epidermal areas of the egg surface, the coat is partly carried into the interior of the embryo and there transformed into the inner linings of the intestinal epithelium, of the neural tube, and possibly of the kidney tubules. In this new position, the surface layer appears to retain its original non-adhesiveness, thereby preventing a fusion of the tubular walls at places where they touch each other. On the other hand, the somite-notochord material which originally forms part of the egg surface seems to lose the coating substance, since after having invaginated it breaks up into tissues which are entirely adhesive. Pieces of gastrula ectoderm, when grafted into the mesenchyme of older hosts, may either retain their epithelial continuity forming cysts, where the coated surface is turned inside, or they, too, break up into cell strands which have no apparent proximo-distal polarity, are adhesive, and disperse within the host tissues.

During the course of development, the coated surface of the epidermis undergoes a progressive hardening, a process which can be demonstrated by subjecting tissues of different developmental stages to the tension at the interface between air and aqueous media having different surface tensions. Such experiments show that the resistance of the coated ectoderm to being torn apart by the interfacial forces increases considerably after the end of neurulation, and that already in younger stages the cohesion of the coated surface is markedly stronger than that of the inner, uncoated surface of the epithelium (Holtfreter, 1943b). Ectoderm cells which are isolated singly in physiological salt solution acquire, within a few days, an entirely rigid and non-adhesive surface. At the same time, their amoeboid motility disappears, while their ciliary movements continue. This solidifying effect of the external medium is further exemplified in those ectoderm cells which, in a normal embryo, move from deeper layers into the outer surface epithelium, where they acquire the characteristics of coated cells.

It seems reasonable to suppose that the progressive hardening of the epidermal surface is largely a result of the progressive formation of insoluble calcium proteinates, since the length of time required to disintegrate the surface coat in a given concentration of potassium oxalate, of sodium citrate, or of sodium chloride, increases with the developmental stage of the embryo. Swimming larvae can no longer be disintegrated by these methods, indicating an insoluble and possibly denatured state of the surface proteins.

Observations on the Properties and Functions of the Cell Membrane

The eggs of various animals, for instance those of some Nematodes (Spek, 1918) and of the trout (Yamamoto, 1940), before or after the onset of the cleavage process, exhibit undulating or rotating movements of their surface. In the oocyte stage, some eggs, such as those of echinoderms, may form pseudopodia of various shapes, particularly under the influence of penetrating spermatozoa, or of chemicals with parthenogenetic properties (Seifriz, 1927; Runnström, 1928; Runnström and Monné, 1945; Harvey, 1938). Intact amphibian eggs do not seem to perform surface movements although, under abnormal conditions, an unfertilized frog's egg may form local bulges and papillae which tend to become pinched off by constriction (Holtfreter, 1946a). This inertness of the amphibian egg may be the result of mechanical restraint exerted by the relatively tough coat. On the other hand, the uncoated side of the blastomeres, facing the interior of a morula or blastula, is normally endowed with filiform or knob-shaped pseudopodia (Holtfreter, 1943b). With progressive differentiation, cell motility increases, as may be seen by the morphogenetic movements and by the subsequent migrations and amoeboid changes of the cells. The following data seem to indicate that it is the external cell membrane rather than intracellular structures which are responsible for these amoeboid movements (Holtfreter, 1946–1947).

Structural Organization of Embryonic Amphibian Cells. The architecture of any cell from early amphibian embryos closely resembles the general organization found in *Amoeba proteus* (Chambers, 1924; Mast, 1926) and in *Pelomyxa* (Wilber, 1946). There are four concentrically arranged layers: an inner core of semiliquid "plasmasol," containing the nucleus, the structural cytoplasm, and all of the granular inclusions. Even in a resting cell, Brownian movement causes damped translocation of the granules. This material is enclosed in a wall of "plasmagel," the viscous consistency of which prevents dislocations of particles embedded in it. The third layer consists of the clear "ectoplasmic fluid" which occasionally contains a few granules derived from the granulated endoplasm. The unrestricted translocations which the granules exhibit when entering this layer indicate clearly its highly fluid consistency. The hyaline fluid is surrounded by a well delineated plasmalemma, or cell membrane, which may fold into wrinkles, suggesting a semi-solid consistency.

The thickness of the hyaline layer varies with the composition of the external medium. Under approximately normal physiological conditions, the fluid accumulates only in regions where the cell surface bulges out into a pseudopodium, and it fades out from view where the cell membrane becomes temporarily apposed to the plasmagel (FIGURE 1). However, although not visible everywhere, this layer seems to form a continuous shell around the endoplasmic granuloplasm. The continuity can be readily demonstrated when the amount of ectoplasmic fluid is increased following immersion of the cell in isotonic saline solutions lacking calcium, or having a pH between 9 and 10. Solutions more acid, and rich

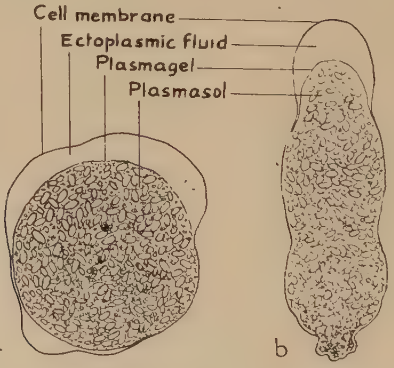


FIGURE 1. Protoplasmic structure of embryonic amphibian cells.

in calcium, have the opposite effect of reducing the hyaline layer. This agrees with observations on amoebae (Pantin, 1923, 1926) and on amoebocytes of *Limulus* (Loeb, 1928).

The cells constituting the surface of the amphibian gastrula possess a proximo-distal polarity which seems an expression of an inside-outside gradient of the egg. When cells from the periphery of the embryo are isolated mechanically, or by means of coat-dissolving chemicals, the former coated side becomes the posterior cell pole which is characterized by a comparatively stronger contractile power of its surface membrane, by the accumulation of pigment, and by the fact that this side has a reduced tendency to form hyaline protrusions. The uncoated proximal side of the cell becomes the region of pronounced amoeboid activity. It represents, therefore, the advancing anterior pole when the cell is resorting to locomotion. Isolated embryonic cells of any germ layer, but especially those of the neural plate, tend to stretch themselves along their proximo-distal axis into cylindrical bodies having an anterior cap filled with ectoplasmic fluid, while the posterior pole is usually marked as a blackish knob showing surface wrinkles. Following exposure of the cylindrical cell to liquefying agents, such as alkali or sodium citrate, hyaline protrusions also appear along the side walls suggesting that there, too, the cell membrane has remained separated from the plasmagel by a thin layer of fluid (FIGURE 2). With further uptake of water, the ectoplasmic fluid forms a spacious shell around the entire endoplasm, followed by a rounding-up of the whole cell into a more or less spherical body.

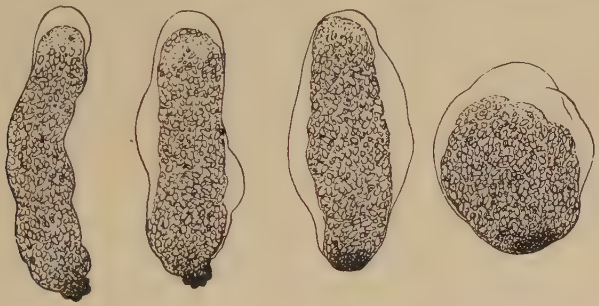


FIGURE 2. Exposure to alkali of a cylindrical cell from the medullary plate causes a lifting-off of the cell membrane from the plasmagel tube and a rounding up of the cell.

Amoeboid Movements. According to the widely accepted theory of Mast (1926, 1941), form changes and locomotion in *Amoeba* result from contractions of the posterior portion of the plasmagel tube, which push the enclosed plasmasol forward against the plasmagel wall bordering at the hyaline cap of a pseudopod. Subsequently, the frontal gel barrier becomes solated, breaks down, and the endoplasm surges into the fluid of the cap. There, the granuloplasm is presently regelated at its surface, thus forming an anterior extension of the plasmagel tube. The plasmalemma of the hyaline cap is assumed to be pushed out passively by the hydrostatic pressure of the forward-streaming axial protoplasm. Repetitions of this cycle would result in a continuous transport of the endoplasm from the posterior into the anterior cell portion, *i.e.*, a shifting forward of the whole cell body. Thus, the essential mechanism of locomotion is supposed to consist of localized and reversible sol-gel formations of the granulated endoplasm, with the cell membrane passively yielding to the local variations of the internal turgor.

In elaborating upon this theory, Lewis (1942) assumed that a conversion of the plasmasol into a more viscous state would automatically result in a contraction of this material. Contractions occurred not only in the posterior region of the cell, but Lewis (1933, 1942) observed in lymphocytes and other vertebrate cells that constriction rings passed in regular intervals over the entire cell surface, traveling in an anteroposterior direction. This peristaltic activity was assumed to reside in the plasmagel tube. Lewis concluded that the translocations of the inner protoplasm resulting from these constrictions, together with localized cyclical sol-gel formations, are the essential mechanism of cellular locomotion.

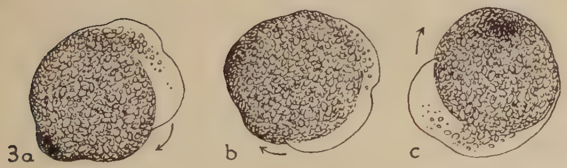
The processes described by Mast and Lewis may occur, likewise, in isolated cells from different stages and germ layers of amphibian embryos. However, the forward-streaming and cyclical sol-gel conversions of the endoplasm observed here, appear to be the consequence rather than the cause of amoeboid movements. Form changes and locomotion of embryonic cells are not necessarily associated with sol-gel transformations of the protoplasm, but seem to be primarily due to alternate expansions and contractions of localized regions of the cell membrane. This conclusion is based upon the following data.

Cellular Motility Associated with Endoplasmic Sol-Gel Formations. Experimental variations of the composition of the culture medium show that both the state of viscosity of the endoplasm and the motility of the cell membrane change with the external conditions, but these two phenomena are not strictly correlated with each other. In a balanced isotonic salt solution ("standard solution") with pH between about 7 and 8.5, isolated cells from the different germ layers of a gastrula or neurula may exhibit the following kinetic phenomena.

Rotating Lobopodia in Spherical Cells. At one or several points of the spherical cell the outer membrane is lifted off the plasmagel and forms hyaline bulges which tend to move around the circumference of the cell, but usually avoid the posterior cell pole. After a bulge has attained maximal size, the underlying portion of the plasmagel may liquefy, and the endoplasmic particles surge into the ectoplasmic fluid where they are freely dispersed and thrown about in rapid motion. Subsequently, the liberated particles undergo a closer packing, become almost immobile, and are refurnished with a capsular plasmagel wall lying closely beneath the cell membrane. Meanwhile, an adjacent region of the cell surface has been growing into a hyaline lobopodium, and the process of solation and subsequent regelation of the inundated portion of the endoplasmic capsule is repeated. Rotating lobopods of a similar kind have been observed in amoebae (Rhumbler, 1898; Jennings, 1904; Pantin, 1923) and in the amoebocytes of invertebrates (Loeb, 1928).

If the embryonic cells are suspended in isotonic saline lacking calcium, or having a pH above 9, they swell considerably through increase of ectoplasmic fluid. A large hyaline cap is formed which rotates rapidly around the surface, while the endoplasmic capsule which is temporarily overflowed by this wave, reacts after a latent period with rapid and merely superficial solations and regelations (FIGURE 3). The belatedness of the endoplasmic eruptions makes it very improbable that this process is the cause of the outbulging and of the rotation of the hyaline lobopod. On the contrary, it seems that the local solations of the plasmagel are of a secondary nature, caused by a liquefying action of the supernatant fluid, the volume of which remains constant throughout the successive cycles of movement. That the cell surface moves independently of the endoplasmic eruptions is borne out by the fact that the larger lobopods consist of several bulges which are separated by notches of contraction, traveling wave-like around the circumference of the cell.

Peristaltic Movements and Locomotion in Cylindrical Cells. The main difference between the pattern of movement in a spherical and a cylindrical cell is that in the latter the hyaline cap and the sol-gel process are confined to the very end of the anterior region. Instead of showing rotating notches of constriction, the elongated cell possesses constricting rings which travel in fairly regular intervals from the anterior to the posterior pole (FIGURE 4). In addition, the elongated cell may perform bending and twisting motions along its main axis and may undergo longitudinal elongations and contractions of the anterior body portion, while the posterior portion is periodically contracted. This pattern of vermiform movements can be exhibited by isolated cells of various germ



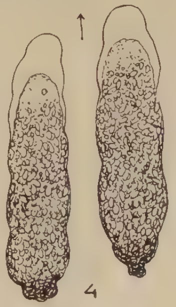


FIGURE 3. Rotating movement and endoplasmic eruptions in a cell isolated from gastrula ectoderm.

FIGURE 4. Peristaltic constrictions and locomotion in a cell from the medullary plate.

layers, although it is most pronounced in the strongly elongated cells from the neural plate.

Since the waves of constriction first appear within the purely hyaline cap, it is clear that they cannot result from a kinetic activity of the plasmagel tube but must be due to an autonomous contractility of the cell membrane. The endoplasmic tube which is, in general, closely associated with the cell membrane seems to be passively molded by the constrictions passing over the cell surface. When a constriction ring approaches the posterior cell pole, the strength of contraction increases, leading to the temporary formation of a small tail knob having a wrinkled surface. The anterior cap never becomes wrinkled but is periodically pushed out in the form of conical bulges. It is these alternating longitudinal elongations and contractions of the anterior region, combined with the periodical shortenings of the tail end which, in the presence of a supporting surface of friction, will produce a shifting forward of the whole cell body. The mechanism of locomotion is thus comparable with that of a creeping earthworm. It is obvious that the peristaltic constrictions, which both in the cell and in the worm occur together with the longitudinal extensions and contractions, cannot be considered as effective movements of locomotion.

It may be mentioned already at this point that the cycle of surface movements may go on indefinitely in the total absence of endoplasmic streamings. Sometimes, however, when a sufficiently large amount of fluid has accumulated in the hyaline cap, the frontal wall of the plasmagel liquefies and the endoplasm flows into the cap where it is presently regelated at its surface. Vital staining of the posterior cell portion shows that, in contrast to the conditions in *Amoeba*, there is no real axial streaming of the plasmasol, but that it is more or less the same circumscribed portion of the anterior endoplasm which is periodically released into the ectoplasmic fluid. This restriction of the gel-sol process to the very tip of the pseudopod is still more conspicuous in the extended processes of more highly differentiated cells.

Cellular Motility in the Absence of a Sol-Gel Cycle. The gelated state of the endoplasm, and hence the frequency of breaks in the plasmagel wall, depend upon the concentration of calcium ions in the culture fluid. Cells which are kept in isotonic solutions of the chlorides of Na, K, or Li, develop a very broad hyaline space, into which the endoplasmic core is almost uninterruptedly releasing its granuloplasm. Under these conditions, regelation of the liberated endoplasm is considerably delayed. It is completely inhibited if sodium citrate or oxalate is applied, either in addition to the chlorides or in pure solutions. These calcium-antagonizing substances inhibit gelation both in hypertonic and hypotonic concentrations. In the former case (0.5 to 2 per cent solutions) the cells shrink and the hyaline space is reduced to a small blister. The endoplasm underlying the blister lacks the smooth surface of a plasmagel wall, its peripheral granules being permanently dispersed in the ectoplasmic fluid. Nevertheless, under such conditions, the cells perform amoeboid movements for several hours.

The independence of the surface movements from the state of viscosity of the endoplasm comes out more strikingly when isotonic or slightly hypotonic solutions (0.4 to 0.25 per cent) of citrate or oxalate are applied. The cells swell in inverse proportion to the concentration of the solution, this swelling being confined to the ectoplasmic layer which may eventually separate the endoplasmic capsule completely from the cell membrane. After a rapid succession of local solations and regelations, the plasmagel wall breaks down all over its surface and the granuloplasm becomes freely dispersed in the surrounding fluid (FIGURE 5). In spite of the total absence of a plasmagel layer, the cell membrane continues performing undulating movements for several hours. This would suggest (*1*) that membrane motility is less dependent on the presence of calcium in the external medium than is gelation of the endoplasm; and (*2*) that the surface movements cannot have been caused by contractions, or axial currents, of the endoplasm, but must be ascribed to autonomous changes of the cell membrane.

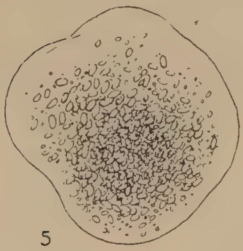
This total liquefaction and dispersion of the endoplasm is reversible when the cells are returned in time to a balanced salt solution containing calcium ions. Then the granules aggregate into a densely packed body and are surrounded again by a gelated wall which may be clearly distinguished from the fluid hyaloplasm. However, prolonged exposure to citrate or oxalate of hypo- or hypertonic concentrations eventually immobilizes and even dissolves the cell membrane. Disintegration of the membrane begins at one side of the cell and spreads slowly over adjacent regions, while the still intact portion of the membrane fails to contract, indicating that the decomposition of the surface film is preceded by a loss of its original elasticity.

Similar observations have been made on cells which were exposed to alkaline or hypotonic media.

Cell Movements Despite Permanent Quiescence of the Endoplasmic Capsule. The reverse of the conditions in the preceding experiments, namely a permanent gelation of the endoplasmic wall, can be obtained by lowering the pH or increasing the relative calcium content of the culture fluid. Although these treatments simultaneously decrease the amount of ectoplasmic fluid, the amoeboid motility of the hyaline bulges or pseudopods is not suppressed.

Another method of demonstrating the autonomy of the surface movements consists in subjecting the cells to standard solution containing alcohol (10 to 15 per cent). This treatment increases rather than decreases the volume of the hyaline layer, whereas the endoplasm contracts into a quiescent and sharply delineated ball which touches the cell membrane only in the posterior cell region (FIGURE 6). In the complete absence of endoplasmic solations, the cell membrane continues performing the characteristic undulating movements which shift the ectoplasmic fluid over the smooth surface of the endoplasmic capsule. It is mechanically inconceivable that any contractions of the capsule, or endoplasmic currents, might have caused the simultaneous appearance of more than one rotating bulge in the cell surface.

Attention may also be drawn to cells of the type of FIGURES 7–9 which show the presence of numerous cylindrical pseudopods, devoid of endoplasm and moving independently of each other. The movements consist in bendings, longitudinal extensions and contractions, and peristaltic constrictions, traveling from the tip to the base of the hyaline pseudopods. These movements cannot be explained by a possible kinetic activity of the encapsuled endoplasm which remains quiescent. Such an outgrowth of the cell surface into numerous blunt pseudopods can be induced by a shock treatment with alkali.



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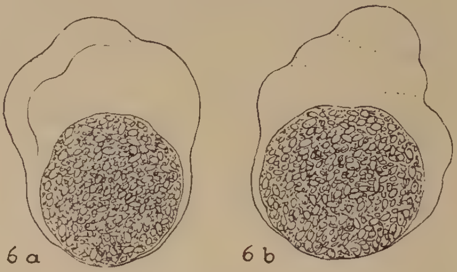

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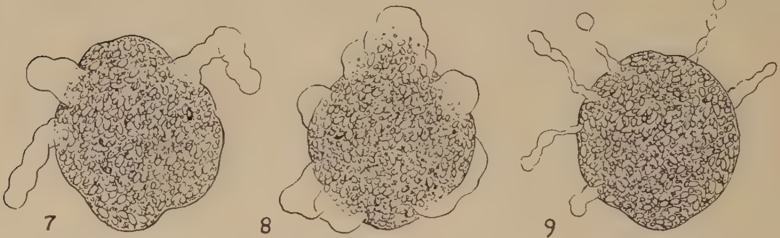


FIGURE 5. Complete solation and dispersal of the endoplasm under the influence of potassium oxalate, while the cell membrane continues to show amoeboid movements.

FIGURE 6. Exposure to 10% alcohol, preventing solation of the plasmagel but not the movement of the cell surface.

FIGURES 7–9. Pseudopod formation and fragmentation induced by brief exposure of the cells to alkali.

Amoeboid Motility in Cell Fragments Lacking Endoplasmic Structures. If the conclusion is correct that it is not the reversible sol-gel process of the endoplasm but varying states of contraction of the cell membrane which cause cellular form changes and locomotion, then cell fragments lacking the endoplasm entirely should be motile as well. This is indeed the case.

An effective way of obtaining fragments of the desired composition consists in exposing cells of gastrula ectoderm briefly to alkali, which induces them to spread on the glass surface. After a while, the alkali effect wears off, the flattened cells contract and leave behind on the glass minute portions of their hyaline margin. Since the endoplasm is usually retracted into the bulky cell portion before the peripheral fragments become detached, the latter frequently contain no visible traces of structural cytoplasm. They may survive for many days and exhibit the various kinds of kinetic patterns observed in whole cells, *viz.*, spreading and locomotion of lamellar processes, alternate expansions and contractions of lobose or cylindrical pseudopods, and peristaltic constrictions passing over the surface of tubular fragments (Holtfreter, 1946a). With the aid of a surface of friction, hyaline fragments are capable of creeping forward like a vermiform cell. In cases where a fragment contains a few lipid granules, these move freely within the vesicle, rapidly dislocated by Brownian movement and by the irregular currents resulting from the undulations of the cell membrane. Whenever a portion of endoplasm is present, it forms, within the ectoplasmic fluid, a delineated body having a gelated surface layer and showing but very reduced translocations of the enclosed granules. There occur all intermediary forms between completely hyaline vesicles and others which are almost entirely filled with endoplasmic material. The former are usually much more motile than the latter.

When exposed to increasing concentrations of standard solution, the hyaline vesicles shrink, their motility slows down and eventually stops. This process may be reversed. In still higher concentrations of this solution, the vesicles die and become filled with basophilic granules, showing unrestricted Brownian movement. They can be further dehydrated by various agents such as acids, or concentrated solutions of calcium chloride, basic dyes, or heavy metal salts, all of which transform the vesicle into a thin, granulated film firmly attached to the glass surface (FIGURE 10).

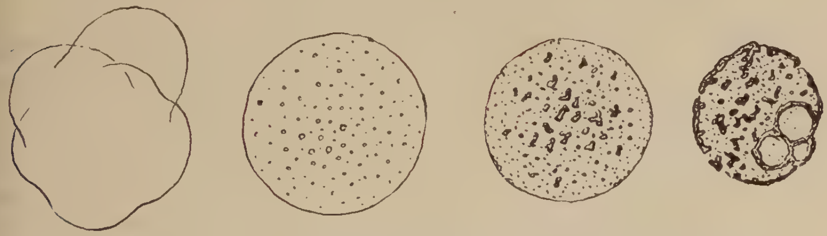


FIGURE 10. Exposure of a hyaline cell fragment to 10^{-1} M $CaCl_2$, causing shrinkage, granulation, and collapse of the vesicle in the form of an adhesive basophilic film.

From these observations, it may be concluded that the different modes of amoeboid form changes can be executed by cell portions which lack entirely the inner protoplasmic material capable of a reversible gelation. It appears that this conclusion applies also to *Amoeba*, for Chambers (1924) observed that cut-off pseudopods of *Amoeba* were capable of ingesting food and moving about in the typical amoeboid manner, although they were entirely free from the visible granules of the parent body. Taken together with the evidence of the preceding paragraphs, these data indicate that the endoplasmic gel-sol formation occurring sometimes in whole cells is merely superposed upon the kinetic activity of the cell membrane. The present material furnishes no evidence that the plasmagel capsule has a contractility of its own. Whenever the endoplasmic material changed from a spherical into a cylindrical shape, showing peristalsis, it was tightly enclosed by the cell membrane, suggesting that it was the well-attested contractile power of the latter which had molded the endoplasm. If these two structures were separated by a cushion of ectoplasmic fluid, the outer membrane continued performing amoeboid movements, whereas the surface of the endoplasm remained immobile.

The periodical solations of the plasmagel apparently result from chemical reactions between the endoplasm and the supernatant ectoplasmic fluid. This process seems to involve the exchange of electrolytes, with calcium ions playing a predominant role in controlling the viscosity of the structural endoplasm. While in some instances liquefaction and dispersion of the granulated endoplasm proceed slowly, comparable to the melting of a submerged sheet of ice containing particles, in other cases the plasmasol may rush rapidly through the opening in the plasmagel wall. This acceleration of flow, in contrast to the liquefaction proper, appears to be caused by the contraction of an external covering. Since forceful endoplasmic eruptions occurred only if the posterior portion of the endoplasm was closely associated with the undulating cell membrane, the contractile force driving the plasmasol toward the hyaline cap is probably furnished by the outer membrane.

Constriction of the Cell Membrane Resulting in Cytoplasmic Division. Isolated embryonic amphibian cells may exhibit three kinds of division phenomena: (*a*) mitotic nuclear division associated with cytoplasmic fission; (*b*) division of the nucleus into two or more nuclei without segmentation of the cell body; (*c*) cellular fragmentation in the absence of nuclear division. The latter phenomenon may occur within a normal embryo, for instance in the flask-shaped blastoporal cells, after they have reached their final destination in the anterior region of the archenteron. The formation of blood platelets has been attributed to the budding-off of cytoplasmic fragments from large mother cells. Lewis (1942) ascribes cytoplasmic division to the same mechanism which produces the constriction waves in migrating cells. The following data support this concept, although they do not agree with the idea of Lewis that it is the plasmagel which performs the constrictions.

In the cylindrical cells mentioned above, it was frequently observed that a constriction wave became stationary, that the constricting ring cut progressively deeper into the cell body and finally caused a fragmentation of the cell. The products of division were usually very unequal in size and composition. The nucleus remained inert and intact and came to lie in one of the daughter bodies, both of which could survive for several days. That it was again the cell membrane which provided the mechanism of constriction was indicated by observations on the cell fragments mentioned above. Very small hyaline fragments containing neither chromatin nor any other microscopically visible internal structures, would divide spontaneously into two bodies which continued performing amoeboid movements.

Cytoplasmic division in the absence of a nucleus has been observed in the eggs of the axolotl, the second spindle of which had been removed by means of a micro-pipette (Jollos and Peterfi, 1923). Fankhauser (1934) observed in polysperm merogons of *Triton* that cell walls may form around astrospheres lacking chromosomes and that segmentation of the cytoplasm may occur even independently of any nuclear or astral activity. On the other hand, division of the sperm nuclei or of the accessory astropheres was not necessarily associated with a corresponding cleavage of the cytoplasm. In centrifuged eggs of the sea urchin which had been activated by hypertonic sea water, Harvey (1938) found that cleavage could take place while the egg nucleus was still intact, which suggests that "the cleavage of an egg and the nuclear changes usually accompanying it are quite separate phenomena." Non-nucleate halves of the eggs of sea urchins and of *Chaetopterus*, which had been treated with a parthenogenetic agent, performed amoeboid movements and then fragmented into cells of irregular sizes containing astrospheres but no chromatin (Harvey, 1936, 1938, 1939). In non-nucleate pieces of the starfish egg which had been subjected to parthenogenetic agents, Chambers (1924) observed the budding-off of fragments, some of which possessed no cytasters. There is no observational evidence that the cytoplasmic fissions occurring in bacteria and some algae lacking a distinct nucleus are associated with the presence of cytasters. These observations make it improbable that cytaster formation is a necessary prerequisite for cytoplasmic division.

It would appear that regional differences of composition of the protoplasm, associated with the presence and division of astrospheres, tend to localize and arrest the autonomous constriction movements of the cell surface in an equatorial zone. Here, the constriction would proceed to form a cleavage furrow, perhaps because the underlying cytoplasm is comparatively more liquid and, hence, less resistant than elsewhere, or because some chemical or electric properties of this region induce a locally stronger contraction of the cell membrane. Chalkley (1935) noticed in dividing amoebae that the plasmagel layer in the region of the deepening furrow becomes thinned out, liquefies, and streams away in oppositely directed axial currents. Erlanger (1897), Spek (1918), and other investigators have recorded the streaming of superficial liquid cytoplasm from the poles toward the plane of cleavage. In centrifuged sea urchin eggs which were subsequently fertilized, the first cleavage furrow sank in more rapidly on the hyaline than on the densely granulated side of the egg (Chambers, 1924).

These observations do not support the view that the viscous plasmagel layer is actively engaged in the cleavage process. According to observations of Motomura (1935) on sea urchin eggs, the external plasma membrane covering the furrow cuts through the viscous cortical layer without dislodging it from the egg surface. Dan, Yamagita, and Sugiyama (1937), from observations on the displacement of kaolin particles attached to the plasma membrane of sea urchin eggs, concluded that the surface area over the spindle poles expands throughout the cleavage process while the prospective furrow region at first shrinks, then increases markedly when it is drawn into the depth of the furrow. Similar conditions apply to the amphibian egg (Schechtman, 1937). However, the opaqueness of the latter egg makes it impossible to watch the behavior of the cytoplasm. That the ingression of the furrow results from the constriction of a densified region of the cell membrane seems to be suggested by the fact that the floor of the furrow is more resistant to chemical and mechanical injuries than is the cell surface over the spindle poles (Just, 1922; Chambers, 1938). This would agree with the conditions in hyaline pseudopodia and cell fragments where it can be noticed that the constricting rings acquire temporarily a higher refractivity than the expanding areas, suggesting alternate states of density of the cell membrane. In dividing fibroblasts, the opposite poles of the cell are known to bulge out into rapidly moving blebs which recall the hyaline blisters formed under the influence of alkali, mechanical irritation, or other agents which weaken and liquefy the cell membrane.

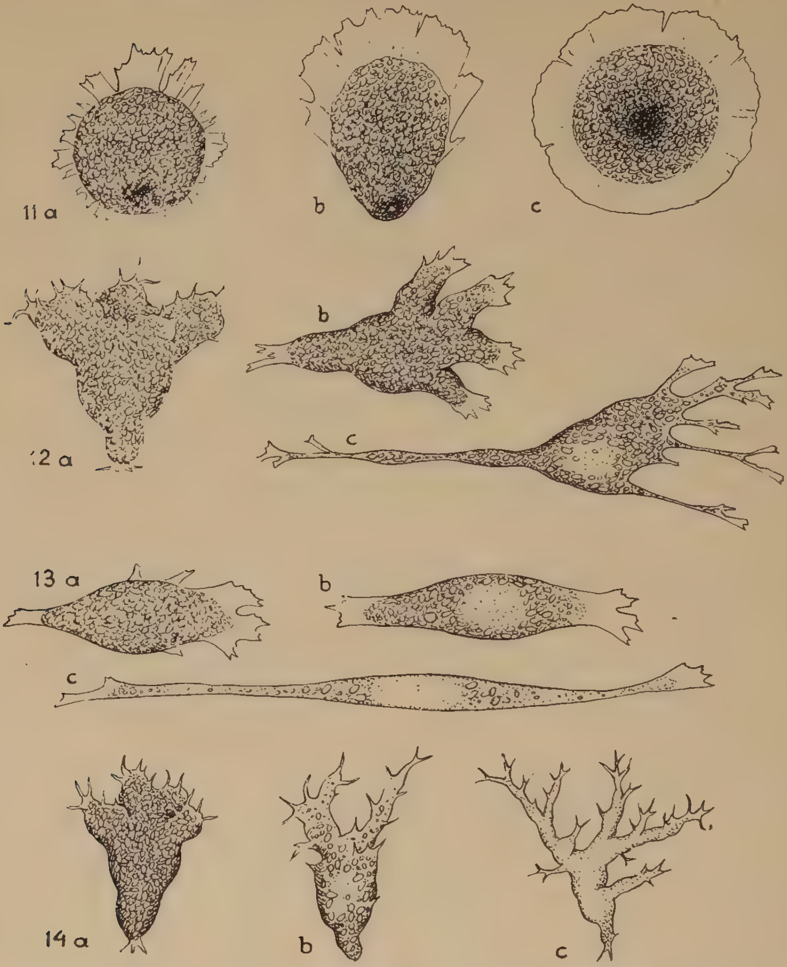
The above considerations make it understandable that external stimuli, causing a local reduction of density and contractility of the cell membrane, will at first increase the permeability of this layer, then induce the formation of pseudopods and hyaline blisters, and finally cause a pinching-off of the out-bulging portions of the cell. This phase may be followed by cytolysis. Most parthenogenetic agents produce cytolysis when applied for longer periods or at higher concentrations. Comparable with the artificially induced formation of hyaline protuberances and their detachment in immature sea urchin eggs (Runnström, 1928), a pinching-off of amoeboid vesicles free of granulated endoplasm may occur in gastrula cells and in early erythrocytes of amphibians which have been briefly exposed to alkali or other agents which at higher concentrations decompose the cell membrane. Mechanical irritation may likewise cause a fragmentation of the embryonic cell. The main difference between the constrictions associated with amoeboid movement and those leading to cell division, or fragmentation, appears to be that the former are reversible and successively involve various regions of the cell surface, whereas the latter remain localized and cut progressively deeper into the cytoplasm.

Function of the Cell Membrane in the Process of Cellular Differentiation. By way of controlling cellular locomotion and cytoplasmic division, the cell membrane plays an essential role in bringing about the morphogenesis of the different types of cells. A differentiating cell acquires its new shape through the elaboration of new patterns of amoeboid activity, a phenomenon usually accompanied by the development of regional, cell-specific differences of adhesiveness of the surface film. In order to materialize these properties effectively, most cells need an external surface of contact. A few examples may illustrate the situation. The cells to be discussed had been derived from urodele embryos and were cultured singly in physiological salt solution (Holtfreter, 1946b, 1947b, c).

Whereas the different cell types from pre-neurula stages are, after isolation, more or less spherical, showing only slight tendencies of spreading, of becoming cylindrical, or forming slender pseudopodia, their morphogenetic behavior becomes more distinctly cell-specific after they have passed these stages. Isolated prospective epidermis cells from a neurula tend to flatten shield-like against glass or living tissues (FIGURE 11), while the individual cells from the neural plate tend to stretch themselves into long vermiform bodies (FIGURE 4). On the other hand, neuroblasts of the type of Rohon-Beard's cells, as well as the cells from the neural crest, shortly after their isolation from early tail bud stages, adopt a shape somewhat resembling that of an *Actinia* (FIGURES 12a and 14a). In the absence of a supporting substratum, their plump anterior portion may project hyaline tapering pseudopods capable of elongating, contracting, and bending around in curves. The posterior cell portion is conical and can change its shape by way of constrictions and longitudinal stretchings and contractions. These cell-specific differences of kinetic behavior are a reflection of the fact that from now on the various cells are determined cytologically.

Evidently, the different cell shapes arise from the activity of the cell membrane, not the endoplasm. The motile filopods of an early neuroblast contain merely ectoplasmic fluid; the anterior portion of the vermiform neural plate cell may exhibit cylindrical elongations, bendings, and constrictions while not underlain by the endoplasm; the epidermis cell spreads and progresses over the substratum by way of extending a hyaline margin which is distinctly set off from the central body of granulated endoplasm.

With the substitution of lamellar or filiform pseudopods for the earlier rounded lobopods, the amount of ectoplasmic fluid is generally reduced. At the same time, the reversible solation of the plasmagel becomes less frequent and is confined to the increasingly smaller areas which directly border at a hyaline protuberance, while the bulk of the



FIGURES 11–14. Successive stages of differentiation in an isolated epidermis cell (FIGURE 11), in a neuroblast (FIGURE 12), a myoblast (FIGURE 13), and a mesenchyme cell (FIGURE 14).

endoplasm remains unaffected. Local outbreaks of a peripheral portion of the granuloplasm have been observed in the margin of flattened epidermis cells and in the tongue-shaped advancing pseudopods of neuroblasts, myoblasts, and mesenchyme cells. The process is readily recognized because of the sudden acceleration of movement which the granules undergo when pouring into the ectoplasmic fluid. It should be emphasized, however, that, as in the case of lobose or tubular pseudopoda which project freely into the external medium, the flattened pseudopods of attached cells move just as well in the absence as in the presence of gel-sol formations.

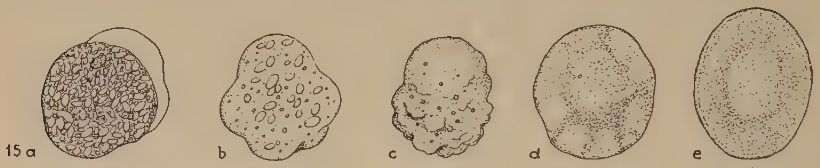
The movements of the cell membrane in flattened cells are less coordinated and on a smaller scale than are those in non-attached cells, where they pass in large waves over the whole cell body. Peristaltic constrictions do occur in differentiating neuroblasts, myoblasts, leucocytes, and mesectoderm cells, but they become less conspicuous the more the whole cell flattens against the substratum. Instead, the hyaline margin of an attached cell is engaged in locally independent and irregularly alternating movements of expansion and contraction which produce a constant change in the outlines of the serrated periphery. Furthermore, ring patterns and ruffles may move over the surface of the hyaline margin. Such ruffles are well-known features of the laminar processes in monocytes and macrophages of vertebrates. Fauré-Fremiet (1929, 1930), who observed them in the freely extended membranous pseudopods of choanoleucocytes, stresses the fact that the movements proceed while the centrally located endoplasm remains quiescent. He therefore rejects the idea that the movements of the hyaline processes are caused by variations of the gel-sol ratio of the endoplasm. This conclusion is supported by our observation that the same kind of movements may occur in isolated fragments of the hyaline margin which contain no endoplasm at all. In migrating cells, the endoplasm merely follows the movement of the advancing margin, by filling up successively the hyaline space, thus securing the ground gained by the activity of the ectoplasmic cell portion. If the total sum of periodical extensions outweighs that of contractions, the flattened pseudopod will continue moving forward in one direction.

All cell types mentioned above possess an antero-posterior polarity. The relatively inert posterior pole corresponds to that side which in the blastula stage was facing the outside. In epidermis cells which are merely spreading, the posterior pole takes up an apical position (FIGURE 11c). In migrating cells, the posterior portion is dragged behind and contracts periodically, frequently forming a tail knob, while the advancing anterior region may remain more or less unipolar (myoblasts) or may branch out into several independently moving pseudopods (neuroblasts, mesectoderm cells, leucocytes). If the posterior pole becomes firmly attached to a substratum and the anterior cell portion continues advancing, the whole cell may become stretched into a long ribbon (myoblasts, FIGURE 13c), or only a tail portion is spun out into a fibrous process (neuroblasts, FIGURE 12c). The transformation of the dendritic anterior protuberances of a neuroblast into long fibrous processes is only possible if the bulk of the cell remains sessile and the pseudopods continue advancing by means of their undulating hyaline end-plate. Leucocytes exhibit a pronounced mobility because of lack of adhesiveness of their posterior region and a great agility of their anterior region.

It appears that, with progressive differentiation, the cell membrane becomes more firmly attached to the underlying plasmagel and that this reduces or inhibits its motility. This condition is especially conspicuous in isolated epidermis cells which have been cultured for several days.

Their entire endoplasm forms a gelated layer closely associated with the surface film, which becomes immobile. In fully extended spindle-shaped myoblasts and in dendritic mesenchyme cells, the major part of the cell surface becomes progressively quiescent, motility being confined to the tips of the pseudopods which have remained free of endoplasm.

Congelation of the whole cell occurs in a pronounced form in erythrocytes (Holtfreter, 1947c). Like all other cells of the amphibian embryo, the erythroblasts represent at first globular cells provided with rotating lobopods which may undergo local gel-sol formation (FIGURE 15a). When the hemoglobin becomes visible and the yolk is almost absorbed, the ectoplasmic fluid slowly disappears and the cell membrane settles down over the entire surface of the plasmagel capsule, without, however, being arrested in its kneading movements (FIGURE 15b). Unlike the spreading movement in epidermis cells, the flattening of the erythroblast into a discoidal body occurs in the absence of any substratum and is executed simultaneously by both the cell membrane and the endoplasm. After the cell has flattened, it remains immobile, and Brownian agitation of the endoplasmic inclusions becomes less discernible. However, the process of immobilization can be reversed, if the early erythrocyte be exposed to liquefying agents, such as salt solutions hypotonic or of high pH (FIGURE 16a–e). Under such conditions, the rim of the discoidal body develops amoeboid protuberances, the cell rounds up and exhibits the kneading movements of its earlier stage, and finally the outer membrane is lifted off from the plasmagel layer and forms once again undulating lobopods filled with liquid cytoplasm now containing hemoglobin. The lobopodia may become pinched off at their base and form mobile fragments (FIGURE 16d). With extended treatment the plasmagel layer becomes liquefied as well, at first locally and reversibly, then all over its surface. This causes the endoplasmic inclusions to be freely dispersed within the now spherically swollen cell. At this stage, the cell membrane becomes immobile and so porous that hemoglobin and even larger particles includ-



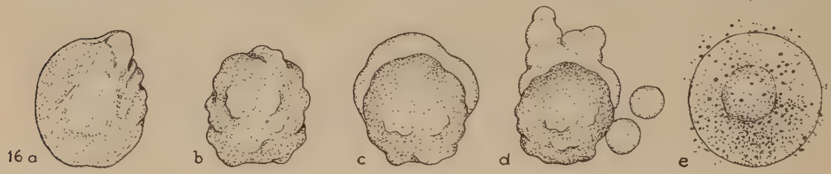


FIGURE 15. Successive stages of differentiation of an erythroblast.

FIGURE 16. Retrogressive form changes of an erythrocyte following exposure to alkali.

ing the nucleus can penetrate it. The final stage of cytolysis is accompanied by the appearance of precipitation granules in the cytoplasm.

A similar sequence of reactions to liquefying solutions can be obtained with other differentiated cells, *e.g.*, leucocytes or mesenchyme cells. The treated cells will return to their earlier mode of motility by replacing the filiform or dendritic processes by large undulating lobopods and they eventually die from being flooded with the immersion fluid.

Thus, cellular differentiation which is associated with partial immobilization and a restriction of amoeboid activity to localized pseudopods, seems to involve a certain amount of dehydration. During the period of consolidation of the final cell shape, the inner cytoplasm may find the proper conditions to develop structures of increasing stability, as, for instance, oriented fibrils. Observational data and concepts as regards the possible genesis of cytoplasmic structures may be found in the publications of Schmidt (1937), Frey-Wyssling (1938), Picken (1940), Schmitt, Hall, and Jakus (1943), and Lawrence *et al.* (1944). If the above considerations be accepted, the appearance of an intracellular cytoskeleton is the consequence rather than the cause of the external form changes in differentiating cells.

Observations on the Composition, Structure, and Physiological Reactions of the Cell Membrane

In order to understand the various functions of the cell membrane, it is necessary to inquire into its chemical composition and physical properties. The pertinent data, although obtained with different methods and on different material, appear to conform sufficiently to be pieced together into a comprehensive, though somewhat sketchy concept. (See the reviews by Harvey and Danielli, 1938; Danielli, 1942; Schmitt, 1941, 1944; Schmitt and Bear, 1939; Monné, 1946.)

Recent investigations afford ample support for the idea, advanced by early workers on cellular permeability, that the cell is bounded by a film containing lipids and probably also proteins. Not only the surface film of the different somatic cells so far studied, but also the outer protoplasmic layer of eggs appear to consist principally of an organized lipoprotein structure.

Runnström and collaborators (1928, 1943, 1945) have shown that, when unfertilized sea urchin eggs are exposed to lytic agents, such as alkali, fat solvents, high temperatures, hypotonic sea water, or merthiolate, their cortical layer bulges out into hyaline blisters and pseudopods which may become detached in the form of "lipoid vesicles." These formations resemble the pseudopods and hyaline fragments in amphibian cells, which arise either spontaneously or under the influence of various cytolytic agents. The cortical layer of the living normal sea urchin egg is positively birefringent in a radial direction, suggesting an ultrastructure comparable to that in myelin formations, where the elongate phosphatide molecules are assumed to form concentrically arranged bimolecular leaflets, the long axes of the molecules paralleling each other and lying perpendicularly to the surface of the body (Runnström, Monné, and Broman, 1943; Monroy, 1946, 1947). Through the action of fat solvents, the birefringence of the cortex may either disappear completely or reverse its sign, becoming a radially negative one. These observations suggest that the cortex contains alternate lamellae of lipid and protein molecules, the latter being extended in a tangential direction and attached laterally to the polar groups of the lipid leaflets (Monné, 1946). It has not been possible to decide whether the entire birefringent cortex, which is quite thick (about 1μ), or only its peripheral portion should be identified with the selectively permeable cell membrane.

Under dark-field illumination, the birefringent cortex of unfertilized sea urchin eggs has a yellow-orange color, changing into white and bluish-grey when this layer is expanding into the pseudopods mentioned above (Runnström, 1928). Monné (1941), who studied the color variations in the protoplasmic constituents of a great variety of cells, concludes that they reflect alterations in the state of hydration of the colloids involved. According to Monroy and Monroy Oddo (1946), the change and final disappearance of color in the cortex of the sea urchin egg is associated with a progressive reduction of the positive birefringence of this layer, indicating a dispersal or dissociation of the constituent lipid molecules, probably because of an increased solvation. It is interesting to note that a disappearance of color and birefringence can be produced not only by fat solvents, hypotonicity and other lytic agents, but also by a slight compression of the egg (Monroy and Monroy Oddo, 1946). Upon return of the egg to normal conditions, the positive birefringence may reappear, although the cortex of compressed eggs fails to recover completely, exhibiting patches of disarrangement of its molecular constituents.

On the basis of these observations, one may assume that the hyaline blisters which can be produced by both chemical and mechanical agents, are the result of a locally increased solvation and porosity of the cell membrane, and the accumulation of water beneath it. In a similar way, the spontaneous local expansions occurring in the cell membrane of amoeboid cells appear to involve a higher state of solvation as compared with that of contracting regions. In a hyaline pseudopod, the out-bulging portions are less refringent than are the constricted ones. Hyman (1917) found that the tip of a pseudopod in *Amoeba* is more susceptible to injurious chemicals than is the rest of the cell surface.

A cortical fine structure comparable to that in the echinoderm egg has been reported to occur in various other cells. Combined chemical, leptoscopic, and polarization-optical studies on the ghosts of hemolyzed erythrocytes suggest that their limiting membrane consists of alternating lamellae of lipids and proteins, their arrangement resembling the molecular structure of the myelin sheath of nerve fibers (Schmitt, Bear, and Ponder, 1936, 1938; Waugh and Schmitt, 1940). A corresponding lipoprotein structure has been assumed to exist in the cell membrane of living embryonic chicken cells (Hobson, 1941), of differentiated ganglion cells of the frog (Chinn, 1938), and of living erythrocytes and spermatocytes of invertebrates (Monné, 1941, 1946). According to Fauré-Fremiet (1929), the hyaline pseudopods of choanoleucocytes are birefringent when in a dehydrated and agglutinated condition. Alcohol and ether, in contrast to acetone, destroy this property, indicating that it is due to the presence of phospholipids. I observed birefringence in the surface layer of living amphibian eggs and in the cell membrane of the different isolated cells and hyaline fragments discussed above, but I have not yet succeeded in obtaining more exact data on this phenomenon.

The concept of an organized lipoprotein structure of the cell membrane is supported by a variety of experimental results concerning the permeability, surface tension, solubility, and electric properties of the cell surface (see Harvey and Danielli, 1938). The concept agrees, furthermore, with the findings on the chemical composition of this structure. Monroy and Monroy Oddo (1946), on the basis of quantitative polarization-optical studies, conclude that the birefringent cortex of the echinoderm egg consists predominantly, if not entirely, of phosphatides and cholesterol. In the red blood cell, practically all the lipids are concentrated in the outer membrane, which constitutes the posthemolytic residue (Erickson *et al.*, 1938). According to the analyses of Parpart and Dziemian (1940), this membrane contains lipids and proteins at a ratio of about 1:1.7, the lipids present consisting almost entirely (82 to 98 per cent) of phosphatides, especially cephalin, and of cholesterol.

The presence of phosphatides in the cell membrane of embryonic amphibian cells is suggested by the observation that this structure is readily destroyed by cobra venom. Increasing concentrations of alcohol render the membrane highly permeable and may eventually dissipate it entirely. Although the cell membrane is less susceptible to the absence of calcium ions in the immersion fluid than is the coat of the amphibian egg, its amoeboid activity and stability seem to depend on calcium bound to the lipoprotein components (see p. 711). Monné (1946) draws attention to the fact that the surface of many cells has been found to be rich in calcium and magnesium and that nucleic acids are also present. He suggests that the phosphoric acid groups of the phosphatides and of the nucleic acids are held together by calcium molecules.

Comparison between the Behavior of Lipid Models and the Cell Membrane

At this point, a comparison between the behavior of the living cell membrane and that of artificial films of lipids and lipid mixtures may promote the further analysis of our problem.

Fauré-Fremiet (1925, 1929, 1930) and Runnström (1928) observed certain similarities between the kinetic, chemical, and polarization-optical properties of the cell surface and those of myelin tubes from lecithin or cephalin. Having found that the hyaline pseudopods of amoebocytes are anisotropic and contain large proportions of phosphatides, Fauré-Fremiet (1929, 1930) suggested that their movements could be interpreted as resulting from localized variations in the degree of hydration of surface layers of oriented phosphatide molecules. The extent of hydration and, consequently, the expansions and contractions of the pseudopods were assumed to be controlled by pH and by the kind and proportion of the cations present.

Some gross similarities between the appearances and kinetics of living cells and those of models of lipids and their mixtures with other substances have been noticed by several other workers (Herrera, 1932; Crile, Telkes, and Rowland, 1932; van Herwerden, 1933). It was, however, the aspect of molecular structure and permeability rather than that of motility of the cell surface which led to the more intensive studies on lipid models. Harvey (1912) produced vesicular bodies of lecithin and proteins which reacted to neutral red, saponin, and mechanical pressure not unlike sea urchin eggs. Danielli (1936, 1945) and Harvey and Danielli (1936) studied artificial lipoprotein films from the viewpoint of elasticity, interfacial tension, and adsorption power. In a series of important contributions, Bungenberg de Jong and co-workers (1932, 1935, 1937) investigated the effects of physical agents, of hydration, pH, electrolytes, proteins, and various other substances on the behavior of lipid models. The concepts on coacervation which emerged from these studies represent a very instructive guide for an interpretation of the structural and functional properties of the cell membrane. No less fruitful for the cytologist has become the extensive work on monomolecular layers, which is connected with the names of Adam, Langmuir, Rideal, and Schulman.

The writer has become interested in lipid models because their reactions to various chemical and physical agents seemed to explain some processes connected with Golgi bodies and cellular vacuoles (Holtfreter, 1946c). At the same time, these experiments disclosed phenomena which appear to promote an understanding of the cell membrane. This dual applicability of the models is not surprising since the structure and functions of the cell membrane are supposed to resemble in many ways those of the lipoprotein film surrounding intracellular vacuoles (Scarth, 1927, 1940; Bungenberg de Jong, 1932, 1935; Frey-Wyssling, 1938). Some of these observations which have a bearing upon our subject matter will now be discussed. The morphological and kinetic behavior of living cells and hyaline cell fragments was compared with that of myelin bodies from crude lecithin, which were exposed simultaneously to immersion fluids of different compositions. The lecithin used was in an advanced state of rancidity and contained admixtures of cephalin, free fatty acids, and probably other lipids. This impurity of the "lecithin" preparation was not considered disadvantageous, since it appears that the biological membranous structures likewise represent mixtures of different fatty substances.

When immersed in distilled water or dilute Ringer, the lecithin developed the characteristic myelin tubes and vesicles which, with further uptake of water, grew in length and diameter. The thickness of the tubular walls and the extent of elongation varied with the initial mass entering into the swelling process. The larger bodies usually gave rise to complex systems consisting of vesicles which included other vesicles and tubes of various size. Small grains of lecithin rapidly became spherical and developed a large cavity filled with an optically homogeneous fluid, while the external wall was thinned out into a film having about the diameter of the cell membrane. In their optical appearance (ordinary and polarized light), these small vesicles were practically indistinguishable from living contiguous hyaline cell fragments.

Myelin vesicles, stretched by glass needles into a long tube and released, either contracted or broke down into several portions which rounded up into spherical fragments. When embedded in olive oil, hydrated myelin formations could be drawn out into very thin fibrillar and anastomosing structures resembling the processes of mesenchyme cells. The surface film of a myelin vesicle was capable of sealing up a hole made by a glass needle, and of restoring its continuity when penetrated by vacuoles expelled from the interior.

Reactions of Myelin Bodies to Variations of pH. In analogy with the behavior of hyaline pseudopods, myelin bodies form tubular shapes only within a medium pH range, acidulation causing shrinkage and granulation, alkalinization, a vesicular swelling, fragmentation, and dispersal of the bodies. Figure 17 shows diagrammatically the relationships between the pH of the immersion fluid and the type of myelin formations which developed at the periphery of a lump of lecithin within the period of an hour. The immersion fluid consisted of distilled water to which HCl or KOH respectively had been added. It will be noticed that the swelling of the periphery increases in direct proportion to the raising of pH. At the lowest levels (pH 2.0–2.2), only a thin surface layer swells into a homogeneous transparent substance. At higher levels, up to about pH 4.0, myelin figures begin to appear, but shrivel up into granules when with further elongation they come into direct contact with the acid medium. Granulation is no longer detectable above pH 4.2. Instead, swelling continues and increases gradually with rising pH. Within the pH range of about 5.0 to 9.0, there are no marked differences in the size and shape of the strongly expanded myelin bodies. At levels above pH 9.4, the tubes become increasingly spherical and may form numerous thin-walled bulges which are pinched off as separate vesicles or may burst and disintegrate (figure 18).

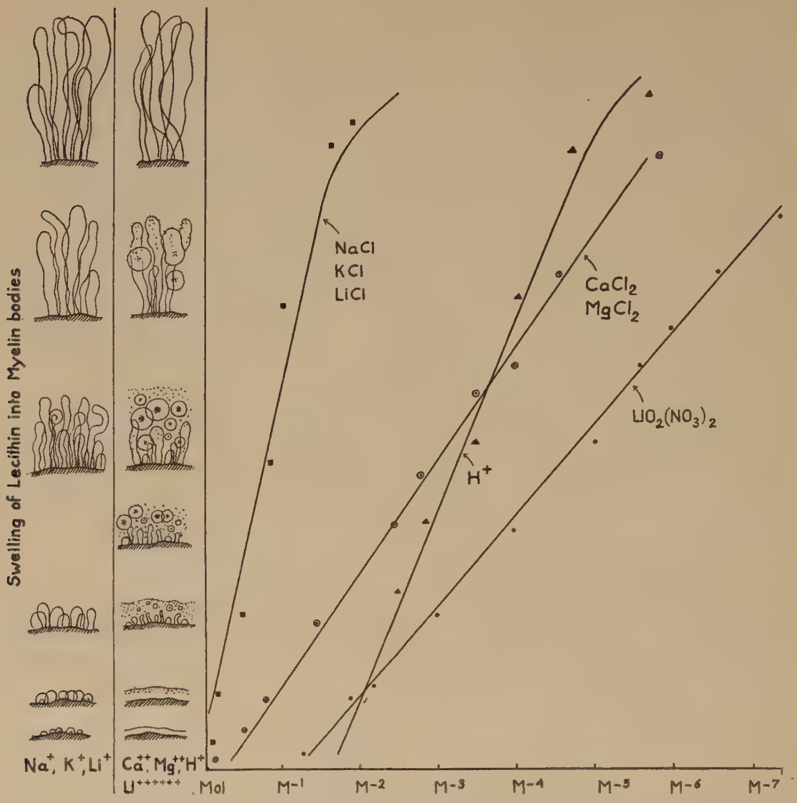
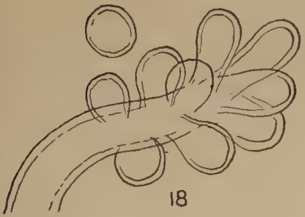
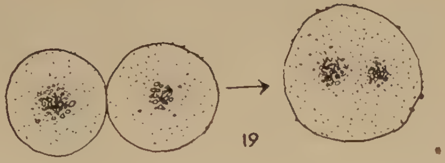


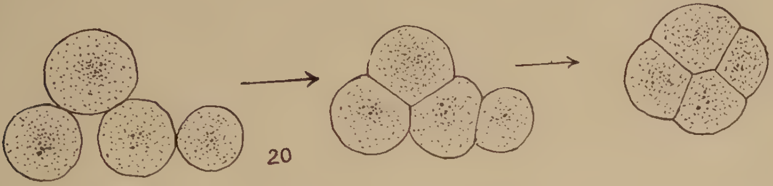
FIGURE 17. Effects of different concentrations of hydrogen ions and various metal ions on the swelling of crude lecithin. The effects have been diagrammatically indicated by the relative thickness of the marginal zone made up of myelin bodies.

When a fully expanded myelin vesicle is transferred from a neutral into an acid medium, it shrinks and the internal fluid gives rise to granules showing Brownian movement. With increasing acidity, the granules become coarser and the vesicle shrivels up and collapses to form a thin, granulated film firmly attached to any contact surface (FIGURE 20a). Adhesiveness of the lecithin vesicles to each other or to glass becomes apparent at about the same pH level (∓4.2) when granules are being formed. The extent of flattening of the bodies over a substratum increases with the rate of dehydration.

In an analogous manner, the size, shape and adhesiveness of the hyaline processes in embryonic cells depend upon the pH of the immersion fluid. In standard solution which is rendered alkaline, the cell swells and its filopodia are transformed into large bulges which may become detached as vesicles and eventually disintegrate. Cellular aggregates break up into single cells at pH above 9.4 and below 4.2. Beyond this range,







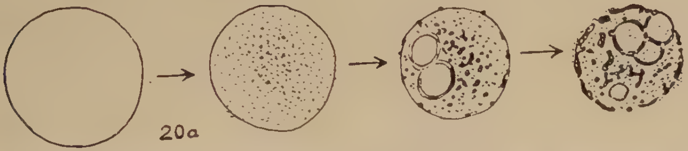


FIGURE 18. Accessory buds and fragmentation occurring in a myelin tube under the influence of alkali.

FIGURE 19. Coalescence of two myelin vesicles in a 5×10^{-4}M solution of $CaCl_2$.

FIGURE 20. Aggregation of myelin vesicles in a 10^{-2}M solution of $CaCl_2$.

FIGURE 20a. Shrinkage, granulation and collapse of a myelin vesicle under the influence of acid or other desolvating reagents.

amoeboid motility ceases, and the cells eventually die. On the acid side of this range, the dead cell membrane becomes increasingly adhesive to glass or to lecithin vesicles, and the content of hyaline cell fragments precipitates into granules. Strong acids cause complete shrinkage and collapse of the fragment into a coarsely granulated film spreading on glass (FIGURE 10).

Effects of Metallic Ions. The rate of swelling of lecithin in solutions of the chlorides of Na, K, Li, Ca, and Mg varies with the salt concentration and with the valence of the cations, bivalent cations having a much stronger inhibiting effect than monovalent cations (FIGURE 17).

In molar concentrations, all chlorides completely suppressed myelin formation. Equimolar solutions of NaCl, KCl, and LiCl apparently had an identical inhibitory effect which was no longer noticeable at concentrations below 10^{-2}M, while that of $CaCl_2$ and $MgCl_2$ could still be traced at concentrations as low as 10^{-5}M. Morphologically, the effect of the various metal ions was similar to that of hydrogen ions. In higher concentrations of the bivalent cations, only small spherical myelin bodies were formed. Length and width of the bodies increased with a decrease of the salt concentration. When expanded myelin bodies were transferred from distilled water to increasingly concentrated solutions of any of the chlorides they shrank, developed vacuolar and granular structures both within their internal fluid and in the outer membrane, and finally shrivelled up into compact bodies or, if attached to a substratum, into a reticulo-granular film (FIGURE 20a).

In accordance with its strongly dehydrating action, calcium chloride rendered myelin bodies adhesive at concentrations as low as 5×10^{-5}M. When, in more concentrated solutions of $CaCl_2$ or $MgCl_2$ (3×10^{-4}M and stronger), two or more lecithin vesicles were brought into contact with each other, they either flattened one against another, forming close-packed aggregations comparable with those of living cells (FIGURE 20) or fused together into a single sphere with a complete disappearance of the separating walls (FIGURE 19). Such fusions could also be obtained with the application of acid.

In a comparable way, the rate of water uptake of living amphibian cells, or their hyaline fragments, varies markedly according to whether the culture fluid contains mono- or bivalent cations. At concentrations which cause complete dehydration of myelin bodies, all the chlorides used transform a hyaline fragment into an adhesive film studded with granules and having the appearance and basophilic properties of a collapsed lecithin vesicle. The embryonic cells attain maximal lobopod formation and amoeboid motility in relatively strong solutions of NaCl or LiCl (around 5×10^{-2}M). Lower salt concentrations cause cytolysis preceded by an extreme expansion, loss of elasticity and partial dispersion of the cell membrane. In pure solutions of $CaCl_2$ or $MgCl_2$, of any concentration, the cells cannot be kept alive for a longer period. At concentrations around 5×10^{-2}M, these salts produce permanent gelation of the endoplasmic wall, slowly moving pseudopods, and finally total coagulation of the cell. Like myelin vesicles, living embryonic cells are non-adhesive in standard solution lacking calcium. The minimal amount of $CaCl_2$ necessary for cellular aggregation in a 0.35 per cent NaCl solution at pH 8.0 is about 10^{-5} to 10^{-6}M.

A process comparable to the coalescence of adjacent lecithin vesicles has been observed in blastula cells of *Amblystoma*. When exposed to standard solution of double strength or having a highly increased ratio of $CaCl_2$, the separating walls of a number of cells disappeared and multinuclear syncytia were formed. A mutual or multiple coalescence may occur both in normal and pathological cells (fusion of gametes; syntrophoblast of the placenta; giant cell formation in cultured tumor cells, etc.). The phenomenon seems to involve the disappearance of a layer of bound water at the interface of the cells in contact.

Several other metal compounds, such as gold chloride, mercuric chloride, silver nitrate or osmium tetroxide likewise strongly dehydrate both myelin bodies and hyaline cell fragments, while sodium oxalate and citrate influence myelinitic growth no more drastically than do corresponding concentrations of sodium chloride. It seems that the dehydrating power increases with the valence of the cation. Experiments carried out with uranyl nitrate showed this compound to be still more effective than the bivalent cations, since its inhibitory action upon myelinitic swelling could be noticed at concentrations as low as 10^{-7}M (FIGURE 17).

Lecithin vesicles which have become completely dehydrated through the action of heavy metal ions can no longer be reversibly hydrated and are more or less insoluble in alcohol and ether. This suggests that the well-known toxic and cytolytic action of heavy metals may arise, in part at least, from their irreversible combination with the phosphatides of the cell. According to Waugh and Schmitt (1940), copper salts at a 10^{-5}M concentration render the envelopes of erythrocytes largely insoluble in organic solvents and insensitive to pH and to the absence of electrolytes. It is thought that the heavy metal ions form cross-linkages with the lipids and proteins of the cell membrane, thereby disarranging the molecular pattern and increasing the permeability of this structure (see, also, Dawson and Danielli, 1938).

Palmer and Schmitt (1941) have analyzed the x-ray diffraction patterns of cephalin emulsions which had been subjected to the action of various electrolytes. $CaCl_2$ was found to be much more effective in reducing the long-period spacings of the lipid structure than were equivalent concentrations of NaCl or KCl. The pronounced desolvating effect of calcium ions was ascribed to their combination with the phosphoric acid groups of the bimolecular lipid leaflets, and the ensuing expulsion of water from between the polar interfaces of the leaflets. A similar mechanism was assumed to account for the desolvating and flocculating action of basic proteins, such as histone, upon phosphatide emulsions (Palmer, Schmitt, and Chargaff, 1941). This concept appears to give a valuable clue to the interpretation of cellular adhesiveness. Schmitt (1941) observed that when small amounts of histone or thorium salts were added to a suspension of red blood cells, the cells were tightly drawn together and formed aggregates. The phenomenon was explained as resulting from an attachment of the introduced cations to the acidic groups of the cell membrane, causing a desolvation and linking-up of the molecules in the adjacent cell surfaces.

Antagonistic Effects of Monovalent and Bivalent Cations. Prolonged exposure of lecithin to molar concentrations of $CaCl_2$ or $MgCl_2$ renders the substance incapable of swelling again in water. However, myelin vesicles which have suffered only a partial dehydration in less concentrated solutions of these salts will swell again when the solutions are diluted with water. The desolvating effect of the bivalent cations can likewise be counteracted by monovalent cations. Thus, moderately dehydrated myelin bodies which have remained unchanged in a 10^{-4}M solution of $CaCl_2$ for several hours will grow out into long myelin tubes when any of the chlorides of Na, K, or Li are *added* to the solution, provided the total salt concentration does not rise above 10^{-1}M. This indicates that the combination of the bivalent cations with the phosphatide molecules of the external membrane is, to a certain extent, reversible, and that the bivalent cations can be expelled and replaced by monovalent cations whose desolvating action is less pronounced. The reverse is equally possible. Furthermore, an outgrowth of new myelin tubes can be obtained when the immobilizing calcium solution is rendered more alkaline, whereas acidulation causes a further shrinkage of the bodies. These reactions may be reversed several times if the salt concentration is kept within a range corresponding to the one tolerated by living cells.

The apparent antagonistic effect of bivalent and monovalent cations upon the swelling of lecithin bodies is actually of a merely quantitative nature since, at sufficiently high concentrations, both achieve complete desolvation. In consequence of the competition of the different cations for a combination with the phosphoric acid groups, lecithin is capable of exhibiting a moderate rate of myelinitic growth in vertebrate Ringer, although the outgrowth is much reduced if the proportion of calcium ions contained in this solution is applied in the absence of the growth-promoting monovalent cations. The repressive effect of high salt concentrations upon the swelling of phosphatide vesicles may account for the absence of a contractile vacuole in many marine Protozoa, and for its disappearance when a fresh-water protozoan is transferred to sea water. As to the readiness with which cations are exchanged between living cells and their environment, reference may be made to the reviews which appeared in the *Symposium on Quantitative Biology*, volume 8 (1940).

Permeability of Myelin Bodies. Swelling and shrinkage of a lecithin vesicle would be impossible if its external wall were not readily permeable to water. Apart from water, a great variety of solutes have been observed to enter the vesicles. The criteria used were internal precipitations occurring under the influence of the permeated substance, or changes of color of the inner fluid.

It is clear from the preceding paragraphs that the different metal ions mentioned above have no difficulty in passing through the interfacial lecithin membrane, their rate of penetration being measured in seconds rather than minutes. Shrinkage and internal precipitations occur in solutions of histone, egg albumen, and various amino acids (Holtfreter, 1946c). Hydrogen and hydroxyl ions may equally well enter into a myelin vesicle. This can be demonstrated by staining the vesicle in a solution containing an adequate pH indicator dye and observing the change of color occurring inside the vesicle after an acid or a base has been added to the solution. Like the histological "acid dyes" (fast green, eosin, orange G, acid fuchsin), the customary indicator dyes were found to enter the vesicles without producing noticeable morphological changes. These dyes do not precipitate with the lipids, hence are not "stored," and diffuse out again when the vesicles are returned to pure water. They act, therefore, quite differently from "basic dyes" (neutral red, Nile blue, toluidin blue, basic fuchsin, methylene blue), which are taken up from very dilute solutions, become highly concentrated both in the wall and in the internal fluid of the shrinking myelin bodies, and cannot be washed out again because they combine with the phosphatide molecules which become dehydrated. Comparable differences of staining affinity for the two groups of dyes are observed in living cells and their vacuoles and other lipid structures. It must be added that the acidity of rancid lecithin changes the color of the permeated indicator dyes according to their pH sensitivity. The pH of the fluid within the myelin bodies used was found to be about 4.8.

The volume changes of the vesicles occurring in the different solutions discussed above cannot be satisfactorily explained by differences of osmotic pressure between the internal fluid and the external suspension medium, but are primarily caused by varying states of condensation of the limiting membrane. Strong concentrations of desolvating chemicals can be observed to cause a shrinkage of successive surface layers which slide over and peel off from the subjacent, less desolvated layers of the multimolecular wall of myelin bodies.* While contracting, the layers exert a marked pressure upon the enclosed liquid, which may escape explosively through a thinned-out and bursting portion of the wall. These observations may account for the fact that the cell membrane of living cells may burst not only in alkaline or hypotonic media, but also under the influence of histone, or basic dyes, which actually desolvate the membrane. It would appear that, analogous to the myelin vesicles, experimentally induced volume changes of embryonic cells are initiated by physico-chemical changes of the cell membrane rather than by osmotically controlled variations of turgor of the inner cytoplasm.

Models of Pseudopods and of Amoeboid Movements. When a fully extended myelin tube is dried on glass and subsequently covered with water, it sprouts out into numerous thin-walled tubules and vesicles (FIGURE 22). This indicates that through desiccation the homogeneous molecular layers of the tubular wall are disarranged and become oriented in new growth patterns when refurnished with water. Corresponding configurations are obtained when a swollen myelin vesicle is at first partly dehydrated by acid and then exposed to alkali (FIGURE 21). Again, the previously homogeneous surface membrane of the vesicle becomes differentiated into regions which vary in their readiness to expand when the conditions of swelling are restored. This results in the outgrowth of

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* The occurrence of sliding and churning movements within the wall of a myelin body, or of translocations of particles attached to its surface, can be explained by the common assumption that the superimposed bimolecular lipid lamellae are separated by "lubricating" layers of water and may expand and contract independently. Such movements may be wrongly interpreted as indicating a liquid state of the entire wall. Similar translocations have been observed in particles attached to the cell surface of migrating amoebae (Schaeffer, 1920) and of dividing sea urchin eggs (Dan, Yamagita, and Sugiyama, 1937), while churning movements occurred in the plasma membrane of denuded sea urchin eggs which had been touched by a glass needle (Chambers, 1938). It is still a matter of argument how many molecular lipid layers may be present in living cell membranes (Schmitt, Bear, and Ponder, 1936, 1938; Danielli, 1942). If this structure should consist of several molecular layers, then the flowing movements observed might involve a superficial layer having only paracrystalline structure and being, therefore, not in a "liquid" state.

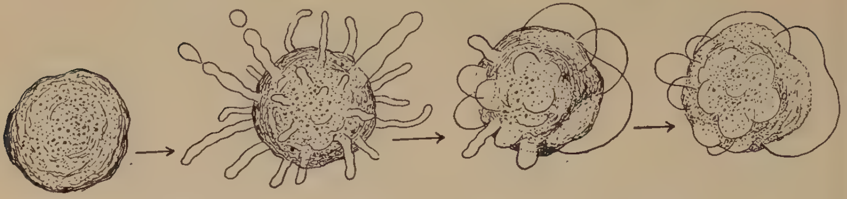
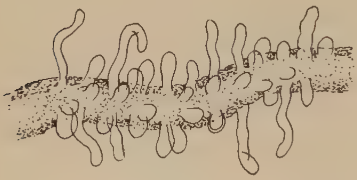


FIGURE 21. Myelin vesicle already shrunk in acid, forms at first tubular, then lobose processes when subsequently exposed to alkali.

numerous processes, the size and shape of which resemble the hyaline pseudopods which are produced in living cells by the application of alkali (compare FIGURES 21 and 9). With a further raising of the external pH, larger areas of the surface film become expanded and the tubular processes change into voluminous bulges which, by way of local and temporal variations in the rate of solvation, constantly change their outlines. Finally, some portions of the swollen periphery may become pinched off in the form of vesicles (FIGURE 21).

Such form changes can be induced by the successive application of many other antagonistic agents which need not have more in common than that the first one has a comparatively stronger desolvating effect than the second one. Instead of using HCl or other acids, one may partly desolvate the myelin bodies by the application of bi- or multivalent metal ions, of basic dyes, or of certain proteins (histone, egg albumen). The subsequent local swellings can be induced by the arbitrary application of alkali, of monovalent cations, or simply by diluting the solution. The process is illustrated in FIGURE 23, which shows a myelin body of the size of a gastrula cell which has first undergone shrinkage



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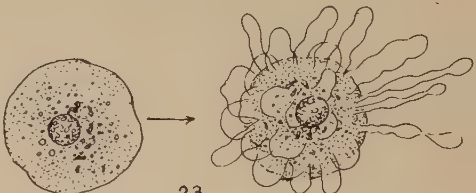

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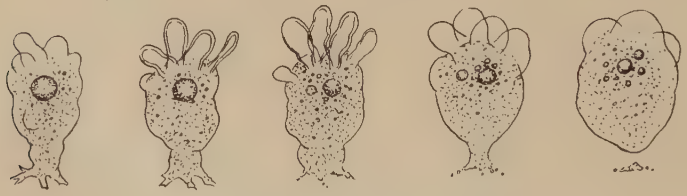

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FIGURE 22. Numerous filial buds growing out from a myelin tube that has been dried and subsequently wetted.

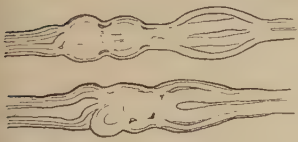
FIGURE 23. Myelin body, partly dehydrated by $CaCl_2$, exhibits tubular outgrowth after the addition of NaCl to the solution.

FIGURE 24. Polar tubular outgrowth in a myelin body containing fatty acid droplets.

through the action of $CaCl_2$, and subsequently expanded into tubular and lobose processes when NaCl was added to the solution.

In addition to the external variables, local differences in the composition of the lipid body may cause the swelling process to become differentiated into a polar growth pattern. This is shown in the example of FIGURE 24, where regional differences of myelinitic activity are evidently correlated to the distribution of droplets of fatty acid which have accumulated in the upper region of the pear-shaped body. The presence of free fatty acids results from the advanced state of oxidation of the lecithin employed. While in fully expanded myelin vesicles the fatty acid particles are, in general, invisibly dispersed, any dehydrating chemical causes them to coalesce into granules and droplets of increasing diameter, which may be finally expelled into the external medium. In the present case, the myelin vesicle had been dehydrated by a strong solution of chrysoidin. The dye rendered the body adhesive to glass and stained it yellow, whereas the coalescing fat droplets stained red. Following the addition of water to the dye solution, the body performed a succession of irregular expansions and contractions, while the surface membrane in the region of the large fat droplet projected a series of pseudopodia-like processes which constantly changed their shape and eventually fused into larger bulges (FIGURE 24). With a progressive swelling of the whole body, the "foot" detached itself from the glass and the fat droplet broke up again into smaller particles which became dispersed in the internal fluid.

Differential swelling of an individual lecithin body can be obtained, furthermore, by introducing into it a particle of a dehydrating substance, such as a basic dye. When the body is immersed in water, the extent of swelling of the different regions increases with their distance from the incorporated particle. However, even in the absence of formed in-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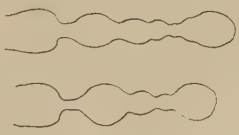
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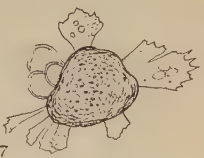
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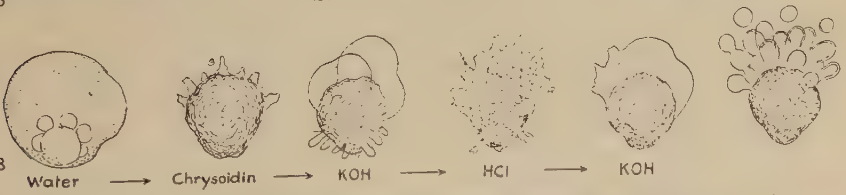

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FIGURE 25. Changing pattern of varicosities in a water-immersed thick-walled myelin tube.

FIGURE 26. Two phases of a constriction pattern in a myelin tube.

FIGURE 27. Membranous myelin processes spreading on glass, following exposure to a strong Nile blue solution.

FIGURE 28. Alternate shrinkage and swelling of circumscribed regions of a myelin body.

clusions, myelin formations may exhibit regional differences of swelling, indicating that the walls themselves may be of an inhomogeneous composition. Frequently, a myelin tube consists of abruptly set-off segments, each having its own caliber and swelling tendency (FIGURE 25). In other instances, the wall appears to be continuous and of equal diameter, yet the body may grow out into side branches, or show along its length an alternation of bulges and constrictions resembling somewhat those observed in pseudopods (FIGURE 26). Although a regular peristaltic progression of the constrictions did not occur, it was frequently observed that terminal portions became pinched off from a myelin tube. As in living cells, fragmentation could be induced by both hydrating and dehydrating agents.

An imitation of "spontaneous" amoeboid movements was observed in the example of FIGURE 29. This myelin formation, while suspended in a dilute solution of alloxan, shrank somewhat and its surface exhibited for a longer period local bulges which were leveled out again and could reappear in the same or in another region of the body. Similar movements of a slightly periodic nature were observed in myelin formations which had been mildly dehydrated by a solution of Nile blue (Holtfreter, 1946c, Figure 16). It may be assumed that the movements were brought about by the antagonism between water and the desolvating agent, the two competing for a combination with the phosphatide molecules and replacing each other alternatingly.

Laminar Processes in Myelin Bodies. Fully hydrated myelin bodies which are non-adhesive to glass, to each other, and to the cell surface, will stick to and eventually spread over these surfaces when sufficiently dehydrated by any of the following agents: alcohol, acids, metal ions, basic dyes, or basic proteins. FIGURE 28 shows the successive stages of transformation produced in a myelin vesicle by the alternate application of hydrating and dehydrating agents. It will be noticed that, when the previously non-attached body shrinks and flattens over the glass surface, its periphery forms a serrated margin. In consequence of dehydration, the lipid components become arranged in new patterns, forming vacuoles, rings, and reticular ridges which can be interpreted in terms of coacervation (Bungenberg de Jong, 1932, 1935). These flowing surface patterns resemble the ruffles and networks which occur in the margin of migrating cells and, in a coarser form, in hyaline fragments that have been transformed into an adhesive film by way of dehydrating fixatives (compare FIGURES 20a, 27, 30c, and 10).

Locomotion of Lipid Bodies. It is well known that, when an oil droplet floating at the water surface is combined with a surface tension lowering substance, such as alkali or lecithin, its spreading is locally increased, resulting in the formation of mobile lobes which may become detached from the periphery. The following experiment illustrates how the local

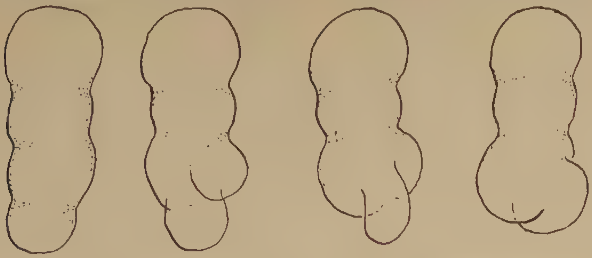

FIGURE 29. Form changes of a myelin vesicle in a solution of alloxan.

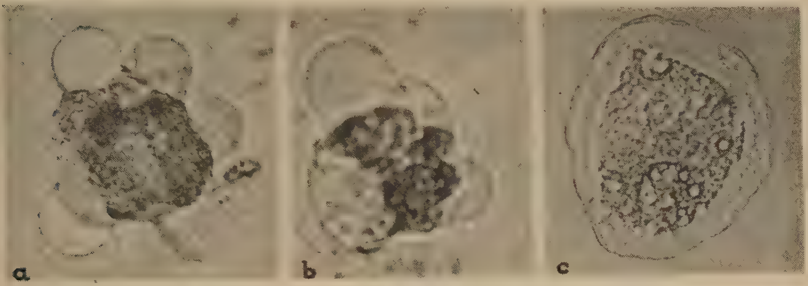

FIGURE 30. Myelin bodies showing hyaline bulges produced by alkali (a, b), or a flattened and adhesive margin with surface wrinkles, caused by acid treatment (c).

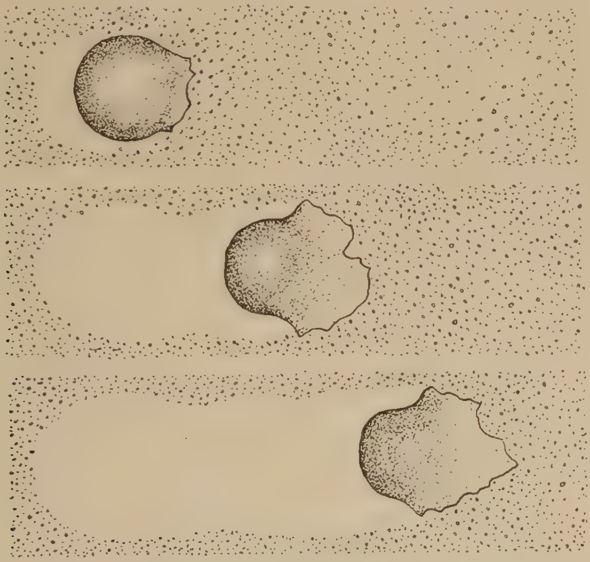

FIGURE 31. A droplet of oleic acid moving through a film of cell debris.

incorporation of protoplasmic constituents may induce a unidirectional locomotion in floating oil droplets.

When an embryonic amphibian cell is brought to the air-water interface, it bursts and releases its content, which spreads as a thin film dotted with fat droplets. The latter originate from the lipochondria, which consist predominantly of neutral fat, phosphatides, and cholesterol (Fauré-Fremiet and de Streel, 1921; Holtfreter, 1946c). When subsequently a small drop of oleic acid is injected beneath this film, it attracts the adjacent lipid components of the film, which are then vigorously absorbed. That side of the drop which is incorporating the surface tension-lowering substances flattens and advances in irregular to-and-fro movements, while the opposite side remains spherical and immobile (FIGURE 31). Thus, the drop assumes the polar shape and kinetic activity of a migrating cell. It moves forward by "eating" its way through the film, leaving behind a trail free of visible particles. Eventually, the whole drop flattens out and becomes quiescent.

Membrane Formation in Relation to Gibbs-Thompson's Law. It is not claimed that the preceding experiment is an ideal illustration of the mechanism of amoeboid movements. However, it shows that the protoplasm of the amphibian cell contains substances capable of inducing surface movements when incorporated into an interfacial layer of another lipid. Previous experiments have shown that the lipid constituents of the lipochondria may combine with the dissolved protein of the yolk to form vesicles bounded by a film which has properties similar to those of a cell membrane (Holtfreter, 1947a). Apart from the granular cell inclusions, the naked cytoplasm of the amphibian egg, when exposed to any kind of hypotonic salt solutions, is capable of elaborating membranous structures in the form of vacuoles or vesicles. It may be assumed that, in normal embryos, the constituents of the cell membrane are recruited from the lipoproteins of the cytoplasm and that the increase of cell surfaces occurring during development is associated with an incorporation of more of these substances into the outer membrane.

Like other surface active substances, phosphatides, obeying Gibbs-Thompson's law, tend to accumulate at interfaces. This can be demonstrated by stirring phosphatide bodies into a water-immersed drop of triolein (Holtfreter, 1946c, Figure 6). The bodies move centrifugally toward the surface of the drop, where they spread as an interfacial film showing the birefringence characteristic of radially oriented lipid molecules. It is perhaps due to the same principle that the pigment granules of the egg tend to accumulate near the egg periphery and along other interfaces, irrespective of their own specific gravity. Other possible examples of this principle are the centrifugal movements of the nuclei in centrolecithal eggs, and the migration of ectoderm cells from deeper layers into the surface of the amphibian embryo. Once the substances of various size and composition have attained a cortical position, they will react with each other and with the constituents of the external phase to form compounds and structures which are absent in deeper layers of the system. The formation of the vitelline membrane and the external coat may be manifestations of such specific interfacial conditions. Furthermore, the pronounced spreading tendency of the ectoderm as well as the proximo-distal polarity of all cells developing from the egg surface are possibly expressions of inside-outside differentials of the egg correlated with the Gibbs-Thompson phenomenon.

In colloidal mixtures, phosphatides not only tend to accumulate at the outer interface, but they may create new interfaces in the form of internal vacuoles. The physico-chemical mechanism and the cytological implications of this phenomenon have been discussed by Bungenberg de Jong (1932, 1935). In connection with this type of coacervation, another phenomenon may be recorded. It was observed that, when the composite lipid extract from lipochondria was spread on glass as a thin layer and covered by a Nile blue solution, it became parceled up into cell-like compartments, differing one from another in their lipid composition, each unit being surrounded by a film of phosphatides (Holtfreter, 1946c). This process recalls the neoformation of cell boundaries in polynuclear syncytia, such as occurs in slime molds, or in the blastoderm of various egg types.

General Conclusions

The wide range of similarities existing between the living cell membrane and artificial phosphatide structures is a challenge to base an interpretation of the functions of the cell membrane upon some of the concepts gained from lipid models. In both the cell and myelin bodies, there was an interrelationship between the phenomena of adhesion, swelling, and motility. When embryonic cells were exposed to conditions which favor the swelling of myelin bodies (high pH, lack of calcium, low salt concentration), they underwent the following changes: loss of adhesiveness, increase of the amount of ectoplasmic fluid, rounding-up of filiform into lobose pseudopods, spreading of amoeboid motility over previously quiescent areas of the cell surface, acceleration of the movements, increase in the rate of endoplasmic eruptions. These phenomena could be reversed by the application of agents such as low pH, hypertonicity, or calcium, which produced shrinkage and adhesiveness in myelin bodies. However, prolonged exposure of the cell to the liquefying agents caused irreversible changes. Amoeboid motility faded out, the endoplasm became dispersed throughout the spherically swollen cell, the cell membrane became flabby and permeable to cell inclusions of increasing size, and finally either coagulated or disintegrated entirely.

Evidently, these phenomena of cytolysis are primarily caused by irreversible changes in the non-lipid constituents of the cell, particularly by a breakdown of protein compounds. An analogy with myelin vesicles exists only in so far as their walls likewise become thinned out and may burst when exposed to alkali. It is probably because of the comparatively greater complexity of the cell structures that their irreversible changes occurring under extreme culture conditions find no true analogy in the behavior of lecithin vesicles. Therefore, a comparison of the two systems should be confined to the conditions which maintain cellular viability.

Polar Structure of the Cell Membrane. The kinetic behavior of isolated amphibian cells showed that their capability of assuming specific shapes develops gradually, but that, independent of their prospective significance, the neurula cells already possess an antero-posterior polarity. This primordial organization was manifested in polar differences of adhesiveness and amoeboid activity, as well as in the tendency of the cells to elongate reversibly into cylindrical bodies. While undergoing further differentiation, the individual cells acquired new and more elaborate patterns of adhesiveness and motility, which determined their direction of locomotion and their tissue-specific shapes. This process was associated with an intracellular digestion of the embryonic lipoprotein inclusions and, apparently, with a certain amount of dehydration.

Observational evidence pointed to the conclusion that the polar organization of the embryonic cell is primarily controlled by the cell membrane. The facts that the posterior pole always represents the originally distal side of the cell, and that the uncoated endoderm cells from the interior of the gastrula do not clearly reveal a polarity, seem to indicate that the polarity originates from the inside-outside gradient of the egg mentioned above. The polar characteristics are partly due to the fact that the posterior cell pole retains some of the properties of a coated cell surface, such as reduced permeability and adhesiveness, pronounced contractility, and the absence of a fluid layer between plasmagel and cell membrane.

However, these considerations do not yet explain the capability of the cell to form cylindrical or actinian-shaped bodies. This faculty must arise from axial differences of structure encompassing the whole cell surface. It has been shown above that, as a consequence of induced local differences of swelling power, the originally homogeneous surface layer of a myelin body may become heterogeneous and exhibit localized tubular growth. Accordingly, it may be assumed that, because of regional differences in the composition of the cell membrane, its response to solvating reagents increases gradually from the posterior to the anterior cell pole, and that in later stages further local differences of condensation of the membrane are developed.

In ectoderm cells, the faculty of adopting a specific shape arises at the time of their cytological determination, suggesting that the process of induction liberates certain compounds, perhaps proteins, which become integrated formative elements of the outer membrane. There is no fundamental difficulty in explaining the various forms of amoeboid movement in terms of solvation of oriented lipid layers of the cell surface. However, it is conceivable that tangentially arranged fibrillar protein constituents of the cell membrane not only determine the elasticity of the cell surface (Harvey and Danielli, 1936) and contribute in differentiating the shape of the cell, but that in cooperation with the lipids they are directly responsible for amoeboid movements. Analogous to myosin micelles, proteins contained in the cell membrane may undergo reversible molecular contractions and extensions. The fact, however, that, in contrast to the lipid models, no protein models have as yet been devised which imitate in any way the various functions of the cell membrane, speaks in favor of the view that the essential kinetic elements of the membrane are the lipids rather than proteins.

Cellular Adhesiveness. It appears that the most important general factor determining, simultaneously, cellular adhesion, permeability, and motility is the state of solvation of the cell membrane. The physiological importance of the mono- and bivalent cations in controlling these phenomena is possibly derived from their readiness of substituting each other in protoplasmic compounds. Adhesion and aggregation of embryonic amphibian cells can be brought about by calcium ions in the ambient salt solution, provided the latter is isotonic and not too alkaline. The bivalent cations seem to operate according to the "zipper mechanism" of Schmitt (1941), by desolvating and pulling together the contacting cell surfaces. This concept presupposes a preponderance of acidic groups, possibly phosphoric acids, at the surface of the cell membrane, an assumption which finds support in the cataphoretic behavior of some cells, particularly of erythrocytes.

While in early embryonic stages only calcium and hydrogen ions seem to be required in order to hold the cells together, an intercellular adhesive matrix, presumably of a protein nature, is reported to exist in between the cells of differentiated epithelia (Gray, 1926; Chambers, 1940). Further insight into the nature of such cementing substances may be gained from investigations of the kind which Chargaff and co-workers (1944) made on the thromboplastic lipoproteins and their function in the process of blood clotting.

The cells in the interior of early amphibian embryos are not firmly aggregated. This is, perhaps, a prerequisite for the execution of the morphogenetic movements which occur during this period. The lack of cellular adhesion apparently arises from the absence, or inactivation, of agglutinating substances in the body fluid, since the cells of the different germ layers will indiscriminately unite with each other when exposed to a balanced salt solution.

With progressive differentiation, there arise cell-specific differences of adhesiveness which are reflected in the display of histotypical patterns of aggregation, disaggregation, migration, and recombination of the various cell strains (Holtfreter, 1939, 1944). It is not known whether these manifestations of a selective adhesiveness result from a molecular lock-and-key mechanism of the naked cell surfaces (Weiss, 1941) or from the interference of specific cementing substances, which may either exudate from the contacting cells themselves or be furnished by the external fluid. Weiss (1947), in a recent discussion of the significance of cellular "affinities" and "disaffinities" for the elaboration of growth patterns, has drawn attention to the similarities which seem to exist between these phenomena and serological reactions. However, aggregations are possible between cells or myelin bodies, the membranes of which may be assumed to have an identical molecular configuration. Nor is it likely that the adhesions occurring between cells and other substances, such as glass, oil droplets, fibrin, etc., are caused by the presence of complementary sets of polar groups at the contact surfaces, comparable to those which are thought to determine antigen-antibody reactions. The ease with which cellular adhesions can be broken up and restored, and the fact that cells migrate in spite of adhesion, indicate that the bonds of attachment are very labile. When observing the gliding movements performed by the surfaces of contacting cells, one gains the impression that the attraction forces operate across an intercalated cushion of water (Holtfreter, 1946b), suggesting that the merely temporary adhesion of migrating cells may be controlled by long-distance forces of the kind which produce the tactoid formation in certain colloidal sols.

Amoeboid Movements. The general rule that cellular adhesiveness decreases with the swelling of the cell likewise applies to the different regions of the individual cell, for the surface of an expanded hyaline bulge is non-adhesive in contrast to the more contracted region of the cell. It seems, therefore, that both non-adhesiveness and expansion of the cell membrane involve a state of increased solvation of this structure, whereas a more contracted and adhesive condition of the membrane would indicate the loss of bound water. The amoeboid movements described above consist essentially of two phenomena: (*1*) periodic expansions and contractions of certain areas of the cell membrane; and (*2*) a more or less regular propagation of these alternate states of activity along the antero-posterior axis of the cell. The first phenomenon appears to be more readily accessible to an interpretation than the second one.

With Fauré-Fremiet (1929), it may be assumed that, analogous to the phosphatide models, the movements reflect periodic variations in the spacing of the radially arranged lipid molecules contained in the cell membrane. X-ray analysis and polarization-optical studies of phosphatides indicate that variations in the water content of these structures not only alter the distance between the bimolecular lipid leaflets but also affect the interchain packing of the molecules (Palmer and Schmitt, 1941). Both in cells and phosphatide models reversible expansions and contractions of the external membrane can be brought about by the alternate application of a great variety of respectively solvating and desolvating agents. However, it is a matter for conjecture what are the intracellular processes that produce the movements in the absence of external stimuli. Probably an indispensable regulative role in the kinetic function of the cell membrane can be ascribed to the antagonistically active hydrogen ions and bivalent metal ions on the one hand, and the hydroxyl ions and monovalent cations on the other. In addition, a reversible interaction between the phosphatide molecules and the cholesterol and proteins present in the cell membrane may determine the extent of expansion of this layer. Bear, Palmer, and Schmitt (1941) have shown that, in the presence of water, the previously incompatible molecules of various lipids (phosphatides, cerebrosides, and cholesterol) will form a homogeneous mixed phase where the different constituents become lined up side by side, showing a single identity period. Monolayers of lecithin undergo a closer molecular packing and, hence, a contraction, when cholesterol is intercalated between the lecithin molecules (Leathes, 1925). In their attempt at interpreting the structure and function of the cell membrane on the basis of coacervate models, Bungenberg de Jong and co-workers (1932, 1935) have pointed to the desolvating and tightening effect of cholesterol, triolein, and oleic acid upon phosphatide structures, this process being strongly influenced by the presence of electrolytes.

As to the effect of proteins upon the packing of oriented phosphatide layers, reference has already been made to the pronounced desolvating action of basic proteins (histone, albumen). However, since the resulting lipoprotein compounds are practically water-insoluble, it appears improbable that strongly basic proteins are engaged in amoeboid surface contraction, which is a reversible phenomenon. More adequate models of the molecular interactions in lipoprotein membranes seem to be represented by the complex monolayers studied by Schulman and Rideal (1937). These authors showed that certain mixtures of cholesterol and wheat gliadin form a composite liquid film on the air-water interface, which gelates on compression. When the pressure is further increased, the film liquefies suddenly, because the gliadin is driven from the surface film into the underlying water. On decompression, the protein molecules reenter the cholesterol film, which expands. This gelation-liquefaction process is reversible several times. It is very sensitive to pH and to the ratio of the lipid-protein concentration. The applicability of these studies to cytological problems is underlined by the observation that cytolytic agents, such as fatty acids or soaps, rapidly penetrate and disperse the artificial lipoprotein films, while such agglutinating substances as tannic acid and gallic acid link the protein molecules together into a hydrophobic "skin" which resists the penetration of soaps.

On the basis of the above considerations, amoeboid movements may be conceived as resulting from localized and alternate states of solvation of the lipoprotein envelope of the cell, these changes being caused by reversible interactions between the radially arranged lipid molecules and other compounds, possibly proteins. The efficiency of this mechanism depends upon a balanced ionic atmosphere. The process may be somewhat analogous to the reversible combinations occurring between numerous enzymes, or pigments, and their carriers, where again lipids and proteins are the most universally present constituents of the systems (Needham, 1942, p. 206; Chargaff, 1944). As to the periodicity and propagation of the surface movements, electric phenomena, comparable to those which accompany the nerve impulse, appear to be involved. Hubbard and Rothschild (1939) made the interesting observation that both the unfertilized and segmented eggs of the trout exhibit rhythmical changes of impedance, of the frequency of about 1.5 per minute. The changes are interpreted as possibly caused by a thickening or thinning of the protoplasmic membrane, involving "a change in the ability of polar or oriented molecules to rotate according to the sense of the applied current."

Summary

Based mainly upon observations on amphibian material, the attempt has been made to show that many embryological phenomena may be better understood if we take into consideration the properties and functions of the interfacial membranes which separate the cells from each other and from the external medium. While all cells are furnished with a living plasma membrane, the periphery of the amphibian egg, and the epithelia deriving from it, possess an additional covering in the form of a coat, which resembles in many ways the hyaline layer in echinoderm eggs. The coat, though not considered to be a living and indispensable part of the egg, plays an important role in determining the viability of the embryo under various environmental conditions, and in controlling, but not causing, the morphogenetic movements of gastrulation and neurulation. This structure seems to consist predominantly of protein compounds, containing calcium, which with progressive differentiation become more densified and less soluble.

The cells derived from the egg periphery possess a proximo-distal polarity which is expressed in polar differences of adhesiveness and amoeboid activity, and in the tendency of these cells to stretch themselves reversibly along an antero-posterior axis. These phenomena are thought to result from regional differences in the composition of the cell membrane, which in turn reflect an inside-outside gradient of the egg connected with the Gibbs-Thompson effect. Subsequent form changes and directed locomotions of the cells are decisively influenced by their inherent axial polarity.

The kinetic behavior of isolated embryonic cells under various environmental conditions indicates that cellular form changes and locomotion result primarily from rhythmic expansions and contractions of the cell membrane. The endoplasmic core, which is more or less separated from the outer membrane by a layer of ectoplasmic fluid, may undergo cyclic sol-gel formations which are, however, not the cause of amoeboid

movements. A cell becomes fragmented if a constriction wave passing over the cell surface becomes stationary and cuts progressively deeper into the cell body. This phenomenon is brought into relation to normal cytoplasmic division.

Local differences of cellular adhesiveness and amoeboid activity, arising in the course of development, are regarded as the main factors which transform the primitive cell into the specific shapes characteristic of the different cell strains. General features of this differentiation process are the replacement of lobose pseudopods by filiform and lamellar processes, attachment of the cell membrane to the plasmagel over the major surface area of the cell, and progressive confinement of amoeboid motility to the tips of the extended pseudopods, which remain free of endoplasm. This process seems to involve a certain amount of dehydration since, on exposure to liquefying solutions, half-way differentiated cells may readopt the appearances and kinetics of earlier developmental stages. After the cell-specific shape has been established, it may become consolidated by the elaboration of an inner cytoskeleton.

The direction of cellular migration, and the histotypical groupings and regroupings exhibited by the various types of cells in a developing organism, appear to be controlled by a selective adhesiveness of the cell membrane, which varies with the developmental stage and with the kind of cells involved. Cellular adhesiveness depends both on the chemical constitution of the contacting cell surfaces and on the composition of the immersion fluid. From the observed antagonistic effects of hydrating and dehydrating agents upon cellular adhesion, it may be concluded that the most universal and essential factor determining adhesion is the degree of solvation of the cell membrane. A prerequisite for cellular aggregation appears to be a certain concentration of hydrogen and calcium ions in the ambient solution.

The attempt is made to interpret the kinetic functions of the cell membrane on the basis of data on the chemical and physical properties of this structure. According to the evidence available, the limiting plasma film of eggs and somatic cells consists predominantly of alternating lamellae of oriented protein and lipid molecules, the latter being chiefly represented by phospholipids. In support of this concept, it is shown that many of the features pertaining to cellular permeability, adhesion, and surface movements can be imitated in models of hydrated phosphatide bodies which are subjected to various environmental conditions. The surprising similarities in the physico-chemical behavior of cells and myelin formations suggest that amoeboid movements result from alternate states of packing of the oriented lipid molecules of the cell membrane. These changes seem to involve reversible variations in the extent of solvation of the lipid leaflets, brought about by the action of electrolytes and, perhaps, proteins and other compounds. The wave-like propagation of the alternate states of film condensation along the cell surface recalls the impulses traveling along a nerve fiber. The above concept would facilitate an understanding of the fact that cellular motility, adhesion, and permeability are usually correlated phenomena and that all three are affected when the cell is acted upon by agents which interfere with the physico-chemical conditions of the cell membrane.

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Discussion of the Paper

Dr. Robert Chambers (*New York University, New York, N. Y.*):

The study of imitations of living processes by the use of non-living material has its good points but requires caution. The plasma-membrane is a structure of which we still know practically nothing. To compare it with a lecithin-like film or even to regard it as a lipoprotein is still a questionable procedure. The plasma-membrane may extraordinarily resemble a lecithin film, but its reaction to electrolytes is quite different. Dr. Holtfreter pointed out that the presence of calcium in the medium stiffens a lecithin film and suggested that this offers still another resemblance to the cell membrane. Let us consider the sea urchin egg. The external component of the surface of the egg can be shown to be an extraneous coat which overlies the "plasma-membrane" and which is removable without detriment to the life of the cell. On the other hand, the selectively permeable and physiologically essential component of the surface is fluid in the presence of calcium and can be shown to be highly liquid when the calcium in the medium is in excess. It is the extraneous coat and not the protoplasmic surface film or the so-called plasma-membrane which is stiffened in the presence of calcium.

Dr. Douglas Marsland (*New York University, New York, N. Y.*):

I was most interested in Dr. Holtfreter's observations bearing on the physiology of amoeboid movement. However, I find it difficult to agree with his conclusion that the force of this movement originates in the membrane of the cell rather than in the plasmagel layer.

One difficulty lies in the fact that the total tension developed in the cell membrane—as measured in a variety of cells, by Harvey, Cole and others—is of a very low order, not in any case exceeding about 5 dynes per centimeter; and such a force does not seem adequate to motivate the movements of the cell. Radical deformation of the shape of the cell by so small a force would not be possible, especially at such times as the plasmagel layer is firmly set—as it is while active movements are progressing. Also, Dr. Holtfreter implies that the force that leads to cleavage likewise resides in the membrane; yet, at the time when the cleavage furrow cuts through the cell, the cortical plasmagel layer of the egg displays a maximum firmness which resists any displacement of cortical granules even by relatively high centrifugal forces.

A second difficulty is that solation and gelation are not visible processes which can be observed directly under the microscope. The hyaline protoplasm immediately subjacent to the cell membrane is capable of undergoing gelation and of developing contractile forces, although Dr. Holtfreter seems to assume that this hyaline layer is always in the state of a sol.

The cell membrane is important, no doubt, as regards the orientation of amoeboid movement, since any local diminution of the membrane tension would be conducive to the outbreak of a pseudopodium at the weakened point. Also, the firmness of attachment between the cell membrane and the subjacent plasmagel must be important, since a pseudopodium can form only in areas where detachment occurs. Such a detachment permits an outflow of plasmasol into the incipient pseudopodium. If a residual layer of plasmagel persists subjacent to the area of detachment, the outflowing sol is sieved free of granules and is perfectly hyaline. However, sometimes the "plasmagel sieve" disintegrates entirely, and then the outflowing sol is filled with granules. But whether it is hyaline or granular, the plasmasol of the pseudopodium has a capacity to gelate, and this gelation guides and supports the pseudopodium and finally limits the extension and permits the pseudopodium to undergo retraction.

The dissociated blastomeres of the amphibian embryo provide an interesting material for the study of amoeboid movement. Free-living amoebae sometimes display a similar set of unusual patterns of movement, but not as frequently or as plainly. However, to discard the sol-gel interpretation of amoeboid movement on the basis of these new observations does not seem either necessary or justifiable.

Dr. Warren H. Lewis (*The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.*):

Little has been said, so far, about the mechanics of development or the forces involved in morphogenesis. Dr. Holtfreter has considered the role of the "cell membrane" in the mechanics of cell movements. I do not agree with some of his ideas, but there is not time to consider them here, as I wish to deal especially with the mechanics of development and morphogenesis which have not been mentioned.

All eggs and cells have superficial gel layers (gel layer for short) which exert continuous contractile tension, a fundamental property of protoplasm when in the gel state. Gel layer and endoplasm are reversible states of the cytoplasm. The superficial gel layer corresponds more or less to the cell membrane of Holtfreter and to the plasmogel plus plasmolemma of the amoeba. It consists of a highly viscous surface layer that shades into somewhat less viscous gel layer. The surface layer corresponds to plasmolemma of Mast and Holtfreter's "surface coat" of amphibian eggs. It is part of the superficial gel layer and might be designated as surface coat. The superficial gel layer plays a leading role in the mechanics of development. Its contractile energy is one of the principal forces involved in morphogenesis.

Local increases and decreases of the contractile tension of the gel layer are responsible for changes of cell form, cell locomotion, flow of endoplasm, cleavage of cells, and (during development) for the cleavage of the egg into its many cells—every cell of the blastula and of even later stages has, for a time, some of the superficial gel layer of the one-celled egg carried into the depths by the contraction of an equatorial band of the gel layer which produces the cleavage furrow. They also account for infiltration and interpenetration by migration of individual cells among others of their own and of different types and for other equally important processes.

When firmly adherent cells of epithelial membranes act together, quite different phenomena result. An increase of the contractile tension on one surface of an area of adherent cells that resist distortion will result in a concave depression, or invagination, on the side of the greater tension. Invagination plays an essential role in early development of blastopore, neural tube, optic vesicle, optic cup, lens, otic vesicle, nasal pit, and probably all other organs that arise from epithelial membranes and tubes.

In addition to cell migrations and invaginations, pulls, pushes, and squeezes produced by contractions of gel layers play essential roles in development.

The zebra fish egg has a strong gel layer that exerts contractile tension in all tangential directions. Contraction of the yolk part squeezes endoplasm out of the yolk to form the disk as its part of the gel layer relaxes. Large yolk globules are held back by a sieve zone. After endoplasm is squeezed out of the yolk, its globules are compressed into polyhedrons by continued contraction of yolk gel layer. During gastrulation, contraction of the yolk part of the gel layer pulls the attached blastodisk over the yolk and at the same time thrusts (pushes) the latter against the disk. As yolk gel layer contracts, it solates and by the time disk edge is pulled to the vegetal pole it has entirely solated. Inner active disk cells migrate around its edge to produce involuting germ ring.

All surface cells of *Amblystoma* blastulae are strongly adherent to one another by their superficial gel layers. The presumptive areas, vegetal pole, dorsal entoderm, ventral entoderm, mesoblast, chordablast, neuroblast, and ectoblast behave differently.

Most vegetal pole area cells migrate inward away from the surface. Some invaginate with adjacent dorsal entoderm at the blastopore and produce a shallow archenteron. As the superficial ends of vegetal pole and dorsal entoderm cells contract, they pull adjacent adherent ventral entoderm toward vegetal pole and blastopore and chordablast to the edge of the dorsal lip. These, in turn, pull mesoblast and ectoneuroblast toward the vegetal pole and blastopore.

The arched dorsal lip contracts and advances and pulls row after row of chordablast cells over its edge against ventral entoderm, like the treads of a caterpillar tractor turning to the ground. This elongates chordablast, extends archenteron posteriorly, covers endoderm (yolk plug), and reduces blastopore size. As chordablast is turned under, it pulls adherent neuroblast toward and finally to the blastopore edge. Neuroblast is elongated and narrowed by this pull.

In the meantime, the superficial ends of the ventral entoderm cells contract and reduce its area. This pulls adherent mesoblast and ectolast toward the blastopore. The superficial ends of mesoblast cells at he mesoentoderm line contract and the cells migrate inward. This pulls neighboring mesoblast cells to this line where they, in turn, migrate inward until all leave the surface. This pulls adherent ectoblast to the blastopore. Mesoderm ingression and dorsal lip contraction result in a contrasting constriction around the mesoderm-dorsal lip circle which s partly responsible for dorsal lip advance and blastopore closure.

These vegetal hemisphere contractions reduce its area and push or thrust yolk mass against blastocoele and ectoneuroblast and help to stretch the latter as it is pulled toward the blastopore.

Archenteron expansion is probably produced by pressure of fluid secreted into it by its lining cells. Secretion of fluid plays an active mechan-cal part in the development of blastocoele, mammalian blastocyst, brain ventricles, optic cup, ear vesicle, etc.

The factors responsible for the superficial gelation of the cytoplasm and for local changes of its contractile tension are unknown.

Dr. J. Holtfreter:

In reply to Dr. Chambers, I should like to refer to what I said with regard to the difficulty of determining by microscopic observation, whether or not such a delicate structure as the plasma membrane has the properties of a liquid (see footnote, p. 739). Apparently, all workers who have tried to elucidate the physico-chemical properties of this structure agree that the outermost layer of living cells is organized in such a way that its molecular constituents cannot move at random, as in a liquid phase. If the concept of a paracrystalline lipoprotein nature of the plasma membrane be accepted, then churning or other movements observed at the surface of denuded eggs should be due to variations in the molecular packing of the organized surface layer. Furthermore, analogous to the translations occurring within the wall of a myelin body, "flowing" motions in the cell surface might be caused by the slipping of a molecular surface lamella over a subjacent lamella. As far as the embryonic amphibian cells are concerned, their living outer membrane appears to have normally a semi-solid consistency. Without the support of the endoplasmic gel wall, it can spontaneously exhibit folds of elastic tension, mobile ruffles, and long tubular or attenuated pseudopodia. This seems to indicate that the membrane is not fluid, and that other forces than surface tension determine its shape and motility. However, prolonged exposure to salt solutions lacking calcium renders the membrane flabby, non-contractile, and highly permeable, whereas, in solutions of a sufficiently high calcium concentration, the membrane shrinks into a definitely solid substance which resists being cut with a glass needle.

With reference to the remarks of Dr. Marsland, I should like to point out that the extraordinary size of embryonic amphibian cells makes it easy to observe their protoplasmic architecture under the microscope. In fact, both the structural and kinetic details in these cells are so spectacular that my undergraduates in embryology have no difficulty in seeing them. Using the well-recognized criterion of the presence or absence of unrestricted Brownian movement, one can readily decide whether the granulated endoplasm is in a sol or gel state. I agree, however, with Dr. Marsland that in more highly differentiated cells, when granulation diminishes and the plasmagel becomes more firmly associated with the cell membrane, these two layers can no longer be optically differentiated, except at the tip of a pseudopod, where they remain separated by a hyaline layer referred to in my paper as "ectoplasmic fluid." Since both this fluid and the plasmagel may be hyaline, their optical appearance is, of course, no criterion for the physical state of the protoplasm.

In the comparatively minute cells isolated from mammals, even such an experienced microscopist as Dr. W. H. Lewis (1942) has not always been able to recognize a gelated cortex, nor could he detect a reversible sol-gel formation when these cells were migrating. Such material is, therefore, unfavorable for an analysis of amoeboid movement. On the other hand, Dr. Lewis has published beautiful microphotographs of the advancing tip of a slime mold (1942, Figures 1–4) which show clearly that in this organism, as in *Amoeba* and amphibian cells, the frontal wall of the plasmagel is separated from the outer membrane by a spacious layer of a hyaline substance, said to be fluid. Furthermore, the outer membrane can be seen to perform lively amoeboid movements while the subjacent plasmagel wall shows a smooth surface, a fact which obviously does not fit into the sol-gel interpretation held by Dr. Lewis and others.

The various patterns of movement occurring in isolated amphibian cells of different age and tissue derivation are not unusual, since they comprise most types of amoeboid movement observed in protozoa and somatic cells. Whether the latter can be interpreted along the lines here suggested remains to be investigated. At any rate, the current sol-gel theory fails consistently to explain the various kinetic phenomena here described, whereas all these phenomena are readily covered by the membrane theory advanced as an alternative. Apparently, the mechanism here proposed is likewise applicable to the interpretation of ciliary movements.

Lack of space prevents me from discussing the ideas of Dr. Lewis on morphogenetic movements. To avoid misunderstandings, I only wish to restate my conclusion that the protoplasmic structures defined respectively as coat, cell membrane (plasmalemma), and plasmagel, differ from each other structurally as well as functionally. In cases where these layers cannot be distinguished from each other, one should not ascribe arbitrarily any function to any one of them which they probably do not perform. I am not aware of experimental data showing that the coat, or any other superficial "gel layer," has essentially more to do with the mechanics of gastrulation than has been mentioned in my foregoing account.

PROSPECTIVE AREAS AND DIFFERENTIATION POTENCIES IN THE CHICK BLASTODERM

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EMBRYOLOGISTS interested in the chick, like everyone else in the field, find an expanding technology opening up before them. In the last decade, even during the war, microchemical, histochemical, and cytochemical tools have emerged and have developed to a degree that makes it immediately possible to obtain accurate knowledge of even the earliest events in development. It is imperative for embryologists to make certain of the definition of their morphogenetic problems, in order that the new tools be applied precisely and profitably. The present opportunity to examine the factual basis of some classical concepts as applied to the chick is thus very welcome.

Germinal Movements during Gastrulation. The discussion will center around problems of the nervous system, commencing with the relationships of the medullary area to the underlying axial mesoderm. It is characteristic of our uncertainty of the course of early embryonic movements in the chick that Dr. Spratt of Johns Hopkins should have been able, just recently, to make us readjust all our ideas of early localization by his marking experiments on the unincubated blastoderm.

It will be well to recall that gastrulation in the chick is performed in at least two steps, perhaps three. The first, which has progressed to a variable degree at the time of laying, is the delamination of the lower layer or hypoblast, from an originally single blastodermal sheet. This step takes place without much superficial morphological indication. The hypoblast customarily is believed to give rise to the entoderm. The mesoderm is invaginated from the upper layer mainly by migration through a morphologically patent blastopore, the primitive streak, which takes shape in correlation with other visible changes in the embryonic area. Descriptions are available of some mesoderm, in early stages, rather delaminating irregularly from the upper layer than invaginating in orderly fashion through the streak. The first appearance of the streak itself presents this picture, which might be considered a preliminary or accessory manner of mesoderm formation.

In view of the current interest in the association of carbohydrate metabolism with invagination, it is surprising that Jacobson (1938) has thus far been the only investigator to describe the invagination or delamination process in the chick as being accompanied by loss of glycogen in the cells involved. This would make the situation comparable to that in the amphibian gastrula, and thus far seems to indicate the only qualitative chemical difference that can be correlated with mesoderm formation.

The primitive streak arises by movement of the epiblast layer to a median posterior line; material is piled up on either side of this line; eventually, a mesodermal sheet grows out underneath, spreading at first laterally and posteriorly. Only after the streak has reached its maximum size does an appreciable anterior migration of continuous mesoderm take place, *i.e.*, the head-process with its paraxial mesoderm. This sheet is preceded by some looser, less coherent mesoderm, the "anterior free mesoderm" or, essentially, the prechordal plate, which has been briefly described by Pasteels (1936) as invaginating also through the anterior streak, ahead of the chorda.

Spratt's (1946) recent study shows with great exactness the movement of carbon marks placed on the surface of the unincubated blastoderm cultivated *in vitro*. His Figure 13 summarizes the information in a coherent diagram: it is clear that the whole posterior half of the unincubated blastoderm eventually becomes mesoderm or streak, whereas the posterior border of the anterior half is the site of a graded but—in peripheral regions—very marked stretching or expansion, considered to be an active rather than a passive process. It would seem that we have at last a definitive answer to the question of how much epiblast material goes into the streak—a question that has received widely differing answers, not only from nineteenth-century workers but from recent experimental workers as well (Wetzel, 1929; Pasteels, 1936). The fact that the work of Spratt was done *in vitro* may perhaps invite objection, but the author controlled his observations by careful measurements, comparing his preparations with normally growing ones, and by many repetitions. If, working in this artificial environment, he had found a less energetic or less extensive invagination than had previously been described, some reservation of judgment might perhaps be justified, since explantation notoriously retards or inhibits many processes. As the case is, however, one feels sure that the displacements are at least as radical as Spratt indicates.

Moog (1944) briefly describes gradients in phosphatase (especially in acid phosphatase) in early blastoderms that might possibly coincide with this concentration of material in the streak. It would be of particular interest to follow the history of regions that evidently expand most actively during this period, to determine if they are the site of enzymatic loss, as might be suggested by her findings.

The disposition of areas of the future embryo in the stage of the maximum or definitive primitive streak is well known from the maps of Wetzel and Pasteels. This plan may be re-projected back on the unincubated blastoderm, with results somewhat as in FIGURE 1. In this figure, the right half shows merely the boundaries of the streak at its inception, nd its definitive stage when the primitive pit, surrounded by Hensen's ode, occupies a position immediately behind the stationary midpoint f the blastoderm. The limit of invagination in post-definitive streak

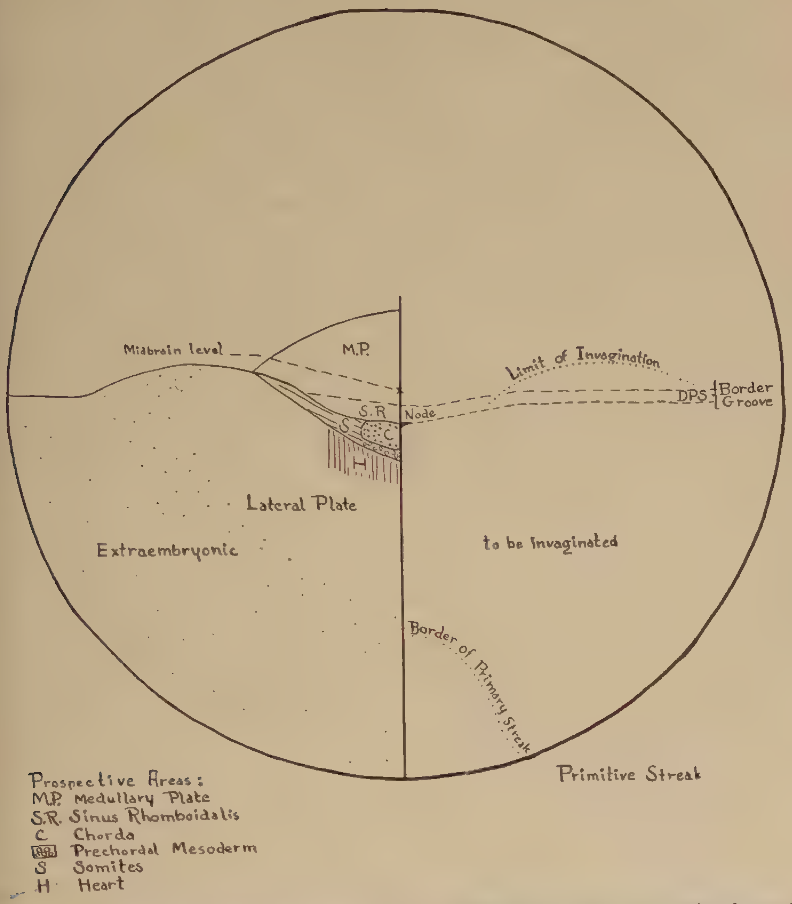

FIGURE 1. Diagram of prospective areas in the unincubated blastoderm, in the light of recent experimental results. Right side shows the primitive streak material, and limits of invagination. Left shows prospective embryonic areas. Intramesodermal boundaries are all hypothetical.

stages has also been estimated from Spratt's 1947 figures. The left side of the drawing attempts to show the organ-forming areas, on the basis of this information. The medullary area must lie anterior to the invaginating region, and the mesodermal areas must enter the streak in inverse order as compared to their final position. There is no accurate experimental basis for boundaries between the mesodermal areas, except possibly in the case of the chorda.

The line between the mesodermal area and the medullary region has a clear basis in Spratt's records. During formation of the streak, the shape of the medullary area must change from something like a half-circle to a long ovoid, largely through stretching and wheeling of the latero-posterior borders. As the head-process grows, the anterior half of the oval will elongate through differential growth greatest in the midline. This process moves Hensen's Node and the primitive streak relatively posterior, and swings segment after segment into rectilinear transverse position. At the stage of the definitive primitive streak, all of the medullary plate that is really laid out, as a flat area, consists of the anterior and lateral parts. The median material, or neural floor, is still to be proliferated from the anterior border of Hensen's Node, along with the notochord.

Thus, different parts of the prospective medullary tube have definitely different histories. Some are older than others, as epithelial areas. The significance of this is evident from the consideration that the impetus toward differentiation of the medullary plate is, in all probability, given by the emigrating mesoderm that comes to underlie the ectodermal layer. The pattern of emigration of mesoderm from the streak is not known as precisely as is the history of the superficial layer, but the general lines can be sketched from morphological reasoning and from the vital stain experiments of Pasteels. The most anterior part of the medullary area is never underlain by more than diffuse prechordal material. The lateral and posterior parts, on the contrary, are underlain by a succession of mesodermal areas, as the sheet migrates laterally: first, probably, extra-embryonic blood-forming mesoderm; then heart and lateral plate; only later parachordal or somite mesoderm takes its definitive station. We definitely do not know the rate or exact directions of this migration, but its occurrence is beyond doubt. As for the neural floor, it seems to be evolved from the front of Hensen's Node *pari passu* with the underlying chorda. The two adhere closely.

Induction of Medullary Plate. The next consideration is a brief review of the evidence, for the chick, that medullary plate arises as a result of induction from an underlying layer. It will be recalled that the original proof of the dependence of the amphibian medullary plate on a stimulus from the underlying archenteron roof consisted broadly of two types of experiment: (1) the test of the ability of carefully specified germinal regions (blastopore lip, archenteron roof or later derivatives) to induce medullary plate in ectoderm that would normally differentiate into something else; and (2) the test of the differentiation capacity of isolated ectoderm, before and after it is underlain by inducing mesoderm. The second test was carried out by various isolation or transplantation methods, culminating in the systematic area-by-area investigation reported by Holtfreter in 1936.

For the chick, Waddington (1930 *et seq.*) has shown that the primitive streak and, to some extent, its derivatives have the capacity to inluce supernumerary medullary plate in ectoderm of widely varying reions of the blastoderm. Woodside (1937) has made clear at what age t is possible to elicit this reaction, *viz.*, response is maximal if the graft ias an opportunity to act while the host streak is developing, and virually disappears when the host is forming neural folds, lingering longer n anterior regions of the blastoderm than in posterior. It is not easy to erify, from published accounts and figures, if every part of the nonnedullary ectoderm has been so tested and has proved capable of formng medullary plate when properly stimulated. Certainly, lateral parts of the *area pellucida* ectoderm and some—presumably all—of that of the *area opaca* can so respond. There seems to have been no critical test of the *area pellucida* ectoderm just anterior to the normal axis, a region peculiar in that, normally, it is never underlain by mesoderm. This test would be of interest. Nevertheless, it seems safe to say that all ectoderm of suitable age can be induced to form medullary plate and to undergo some regional differentiation.

As to the efficient stimulus, the very early streak seems not to have been tested for inducing capacity; but different parts of later streaks, and of the head-process with overlying ectoderm, have proved effective. The complexity of the inductions produced by these non-homogenous transplants acting in the growth field of a host embryo *in vitro* is extreme. This is perhaps clearest in the long series of cases reported by Waddington and Schmidt (1933), which were also heteroplastic (duck to chick and *vice versa*). In this report, the cases in which unmistakable regional character or even polarity can be ascertained in the induced medullary plate are relatively few. From these few cases, however, the authors have shown some evidence for differences in character of the induction, depending on the region (anterior or mid-streak, head-process, etc.) from which the graft was taken.

There is, thus, satisfactory evidence that medullary induction can occur in the chick ectoderm. We may proceed to the evidence that this process occurs normally. For the chick, in the stages when ectoderm is being underlain by mesoderm, separation of the two layers for experimental tests has not been technically possible. The critical, direct test of differentiating capacity of the same region of ectoderm, before and shortly after mesodermal contact, has not been performed. Only indirect approaches can be cited and they have led to new questions rather than to satisfactory answers.

Some time ago (Rudnick, 1938a), separate pieces of the early streak blastoderm were grown *in vitro*. In the earliest series, cuts were calculated—by the information then available but quite coinciding with Spratt's new data—to separate the forming streak, visibly invaginating, from superficial mesoderm about to be invaginated, and these in turn from ectoderm. No embryonic axis differentiated from any piece, but merely little clumps or vesicles of tissue. Few tissues could form after this drastic partition, namely, generalized tissues like blood, mesenchyme, epithelium, and, in addition, heart muscle and medullary plate. Medullary plate, or rather little tubes or spheres, developed from the most anterior (ectodermal) explants in a certain percentage of cases. It is not certain whether they did so from other pieces. At this stage, before the groove of the streak had appeared, it seems impossible that mesoderm invaginated through the streak region could have reached the anterior piece, which, however, contained all or most of the prospective medullary material. In face of this, one is reduced to two radically different alternative hypotheses: either the prospective medullary region, *in toto* or in part, contains potencies for medullary differentiation antedating mesodermal induction; or, in pieces cut and explanted, there is some irregular invagination or substitute therefor, and this process is adequate for induction. Further along this second line of thinking, there is the question of the normal prechordal mesoderm—whether perhaps its regular route of invagination is not by irregular delamination, which could have occurred in the isolated explants; in which case the medullary tubes would represent forebrain.

In this material, there was no indication as to what region or level of the axis the medullary nodules might represent. Even in explants grown from later streaks, from regions where induction by emigrating mesoderm was possible, no regional differences could be ascertained. Clearly such fragments present drastic mechanical opposition to regional morphogenesis.

Spratt (1940, 1942), a short time later, reported experiments in which regional organization had been given a much better chance. These consisted in dividing early blastoderms (pre-streak through head-process or later) into only two parts by a transverse cut. If the cut was made approximately through the midpoint of the blastoderm, in pre-streak or early streak stages, it is obvious that all or part of the prospective medullary plate would be isolated from the intact mesoderm-streak region behind. From cultivation of both pieces, Spratt obtained typically a fairly good axis from the posterior piece in which the streak formed, and a little nodule of medullary plate in the center of the anterior piece. At the time, Spratt interpreted the rather shapeless anterior bits as forebrain, and, in a few cases, on subculturing, obtained retinal pigment from them; the posterior axes he thought specifically deficient in forebrain. One wonders if, in view of his recent localization experiments, he might not wish to revise this view and think of the posterior axes as being inhibited but not necessarily lacking in any one level. It is clear that not only the prospective forebrain but a good bit besides was included in the anterior piece, whereas very little destined medullary material was present in the posterior piece. In development *in vitro*, the anterior cut border of the posterior piece (where lay most of the prospective medullary material that was included) remained fixed to the clot. This emphasizes the conclusion that the medullary plate of the posterior axes must have formed largely from epithelium that normally would have entered the streak to become mesoderm. It would seem that Spratt has shown here, among other things, that prospective mesoderm also can be induced to form medullary plate.

The anterior pieces again present the problem of whether and by what the medullary nodules were induced. To illustrate the difference in result, when essentially the same transection experiment is performed after the whole brain-forming region is underlain by mesoderm, one should consult the figures in Spratt, 1940 (Plate 2). In these cases, the anterior piece clearly complements the posterior, and the mosaic character of the separation of forebrain or its parts from the rest of the axis is obvious.

Thus, attempts to test parts of the medullary field before formation of the mesoderm have served to demonstrate our ignorance of the normal course of invagination, not to mention our ignorance of the organizing substance itself. If, at first glance, things look as if the early medullary field in the chick (the site of the later forebrain) possesses some powers of self-differentiation, it is not likely that such an explanation will satisfy investigators or be allowed to remain without further analysis.

Regional Organization of the Medullary Plate. The forebrain problem raised in the preceding section may serve to introduce some discussion of the pattern present in the early medullary plate. In searching the literature for cases where a forebrain has been experimentally induced, one finds exactly one instance where an indubitable prosencephalon with optic swellings is figured, as contrasted with vague designations of "head" or "head-fold" which usually look very unlike any part of a normal chick brain. This case is shown in Waddington and Schmidt, 1933, Figure 10, and the beautiful induced head lies beside the normal one at about the same antero-posterior level. The transplant performing the induction was from a chick: the anterior half of a medium (*i.e.*, pre-definitive) primitive streak, including Hensen's Node (whether any material anterior to the node was also included, is not stated), placed ventral side up in the right middle region of the duck host, beneath the epiblast. If anything, the induced brain is farther advanced than the host, that is, optic swellings are clear. The transplant itself has differentiated into neural tube and a little unspecified mesoderm. The graft material immediately touching the induced optic swelling is neural tube, which is actually continuous with the host induction. The authors also note that the most anterior part of the induction is not underlain by any graft at all. It seems questionable as to whether the regional character of this induction is to be attributed, as the authors do, entirely to the character of the graft. Its position with relation to the host embryo is too parallel. If forebrain is induced by graft neural tube, as the morphological relations appear to indicate, one would think that the regional character of the induction must be attributable, at least in part, to host influences. Unless pre-nodal material were included in the graft, the normal inductor for telencephalon was not present. The evidence offered by this case for localization within the inducing system is thus very unsatisfactory.

In the head-process blastoderm, each level of the medullary plate is capable of differentiating into a characteristic brain-vesicle, with accurate histological pattern of cell types and layers, when suitably transplanted, along with underlying mesoderm (Rawles, 1936). This antero-posterior pattern also seems to be present in the definitive primitive streak stage, if allowance is made for the condensed nature of the material. In preceding stages, especially in those where it is known that the definitive relations of axial mesoderm to medullary ectoderm have not been attained, no experimental evidence is available. With our current increase of understanding of the history of movements and relative position of the two layers, a real experimental test of their separate roles in the emergence of the antero-posterior pattern will be possible. The evidence found by Spratt, of potency of at least a cellular order to form retinal pigment in anterior ectodermal pieces isolated from the rest of the axis in pre-streak stages (1942), is a suggestion that the idea of pattern in the ectoderm itself must not be neglected.

Transverse organization of the medullary plate, which changes to dorso-ventral order in the embryo, is a process completed, in the cases studied, later than that of the antero-posterior axis. The cases studied are those of the eye (Clarke, 1936) and the neural crest as indicated by pigment cells (Rawles, 1940; Ris, 1941). In both instances, the histological potency to form these lateral or dorsal structures is retained by the isolated median or ventral strip some time after the formation of medullary folds. The exact movements of material in these cases have not been followed. There is a slight chance, for the eye, that a tenuous strip median in the anterior medullary plate, too narrow to be tested by current methods, may, from the first, be unable to form eye tissue, and that the diencephalic floor arises by enlargement of this strip. The situation looks much more like a gradual separation of a continuous eye field into two laterally placed ones by the actual loss of ability of the medial cells to perform a certain histogenetic task. The case of the neural crest melanophores is clearly of this type. This bilateral or dorso-ventral pattern stabilization seems to be a much slower process than medullary induction *per se*. In the amphibian forebrain, the mesoderm has been shown to play a critical role. This would certainly require some localization of pattern in the mesoderm, even if its expression in the ectoderm emerges only slowly.

A morphological basis for a medio-lateral pattern in the invaginated mesoderm is more immediately clear than it is for an antero-posterior one. It has already been pointed out that the lateral parts of the prospective medullary plate are underlain by a progressively migrating mesodermal sheet and are, thus, subject to contact with a succession of mesodermal areas. In the midline, ectoderm and mesoderm are at first massed, without epithelial relations, in Hensen's Node, and grow forward together from that position. This process has been compared to the growth of a tailbud blastema. It is probable that intermediate gradations between these differing processes occur in intermediate regions. Thus, with reference to time and pattern of contact between the two layers, a medio-lateral differential is ready for analysis by more refined transplantation and by cytochemical methods. Furthermore, at the time these patterns are being realized, excised areas can be regenerated from surrounding tissue—even the whole of Hensen's Node (Waddington, 1930 *et seq.*). This offers another means to study the time required for acquisition of new potencies or for the loss of others. These suggestions are made in order to emphasize that, in spite of some of its drawbacks as experimental material in early stages, the chick embryo has definite morphological features which, once understood, promise to make it especially valuable for study of some general problems of vertebrate pattern.

Differentiation Mechanisms. It would seem convenient to distinguish three types of activity concerned in the differentiation of a nervous system from a medullary plate: (1) secretory, responsible for the maintenance of the various cavities of the central nervous system; (2) mass-movements within the whole epithelium, or large groups of its cells, responsible in the first place for closure and shaping of the neural tube, subsequently for formation of layers, cell columns, nuclei, etc., within definite parts of the medullary tube; and (3) differentiation of individual cells in various directions, such as neurons of several types, supporting elements, ependyma, etc. The first activity has not been the subject of adequate study, though the chick embryo would seem to offer an excellent object in which the nature of the barrier between tissues and cavities could be investigated. It is proposed to consider the second and third processes here, briefly, with reference to possible relations one to another.

The induction reaction itself is, of course, of the nature of an epithelial reaction, first visible in the marked heightening of the cells of the induced region. Subsequent processes are also related to the properties of an epithelium, not to single cells; *e.g.*, the changes in form and polarization responsible for the closure of the neural tube. Hobson's (1941) observation that closure, previous to actual fusion of the medullary folds, can be reversed neatly by dehydrating agents such as a drop of glycerine, calls for further analysis. In later stages, the formation of cell layers, so characteristic of various parts of the brain and neural tube, must also be regarded as a series of mass patterns within an epithelium.

As a contrast, we have the cellular differentiation that has been long and closely studied, whereby single cells apparently undergo the characteristic changes involved in forming neurofibrillae and putting forth nerve processes which then acquire peripheral connections. Barron's (1946) recent interpretative study of differentiation in the motor area of the brachial region of the spinal cord may be cited. He finds that certain individual cells of the neural epithelium of the basal plate start differentiation *in situ*, but that this process is correlated with a peripheral migration to the position which will be occupied by the ventral horn. The first pioneer fibers of the ventral root come from such *primary neuroblasts*. In addition, other cells migrate out from the epithelium in an apparently undifferentiated condition, and later become *secondary neuroblasts* by differentiation in striking proximity to the dendritic processes or cell bodies of the primary neuroblasts. Hamburger and his students (*cf.* Hamburger and Keefe, 1944) have already formulated the idea of a cell-to-cell induction, which Barron supports on morphological grounds, as a result of their experimental studies on altering the peripheral load of the brachial or lumbosacral region of the cord. They have shown that the marked development of the motor columns in these two regions is directly dependent on the presence of a periphery with which the motor fibers leaving the cord may connect, and not on any central connections outside of the few segments comprising the limb area of the cord (Hamburger, 1946). Furthermore, the periphery controls the development of the motor columns not by regulating cell divisions or number of cells within the cord, but by controlling cell differentiation, *i.e.*, the formation of motor neuroblasts in the ventral horns. A cell-to-cell mechanism, whereby a pioneer neuron, once it connects with the periphery, puts out one or more dendrites and simultaneously becomes capable of inducing neighboring undifferentiated cells to follow its lead, until the periphery is loaded, provides a most attractive hypothesis, inviting direct experimental investigation. The only lack is a suggestion of what initiates the process in the first place.

The polarization miscroscope studies of Hobson (1941) form a very interesting beginning of optical studies of the neural epithelium just before and after closure of the tube. The indication is of an orientation of protein micells in the long axes of the cells (that is, radiating out from the center of the tube) and lipoid components perpendicular to the protein. A great share of the birefringence was observed in the various membranes (limiting, cell, and nuclear). This particular type of orientation is by no means peculiar to neural epithelium in the chick embryo. It would, however, be most interesting to make similar observations in slightly later stages, when there is more zonation in the neural wall and the pattern of cellular emigration that has just been discussed is better established. It might, thus, be possible to detect differences in content or structure of the migrating individual cells.

Moog's (1943) studies of the phosphatase distribution at various stages of the developing spinal cord show some striking patterns, some of which, such as the median band of alkaline phosphatase, seem very difficult to understand. The preparations are not figured or described minutely enough to ascertain differences of individual cells, so that it is impossible to say if phosphatase gives any promise of being involved in the differeniation under discussion. The program will evidently be to continue to ook for cytochemical and cytophysical differences between individual ells in the neural tube, until the specific difference between migrating nd non-migrating individuals becomes apparent, as well as subsequent ifferences between neuroblasts and undifferentiated cells. A cytochemical cell lineage of the elements and derivatives of the neural epithelium is a highly desirable goal which, when available, will perhaps offer the clue to the mechanism or mechanisms involved.

It is possible that the two major activities in neuron formation—emigration and putting forth nerve processes—are merely steps in one single course of surface changes. Under this view, a series of events would be early initiated in all cells of the neural epithelium, which would progress more rapidly in some cells than in others. In cells changing most rapidly, some double threshold would be crossed and those cells would become primary neuroblasts, simultaneously undergoing internal rearrangement, changing external form, and losing contact with their neighbors to slip outside the epithelium. In cells where the process moves somewhat less rapidly, only an intermediate threshold would be attained. Surface changes would go only so far as to permit these cells to migrate out of the epithelium in a generally rounded state, requiring a further stimulus from outside—an induction—to become neuroblasts; these would be the secondary type. Large groups of cells in which either threshold was reached at the same time would provide a basis for cell layers, columns, etc., as in the differentiating brain epithelium.

It would be well to note that these formal relations within the epithelial structure of the neural tube do not hold any exclusive key to differentiation of neurons. Nerve cells may differentiate in transplanted material that has never had an opportunity to form a tube or more than a most irregular and distorted epithelium (Rudnick, 1938b). It is only necessary to consider the neural crest to realize that no epithelial history at all is requisite to neuron differentiation, however important it may be for the larger pattern of the central nervous system.

If it seems that the foregoing review has been more concerned with exposing our ignorance than with recording progress in the understanding of developmental processes in the chick, it can certainly be replied that the morphological task before us is a most exacting one. We are really faced with ascertaining individual cell differences and cellular relations in material in which, until recently, one cell seemed to look just like another. The fact that all cells in the blastoderm or in the neural tube do not behave alike has led to various hypotheses concerning the mechanisms responsible, *viz.*, progressive internal change; cell contact with chemical or physical effect; even production of substances and their transfer over some distance. The sorting-out of these possibilities inevitably requires more and more detailed knowledge of cellular relations in normal development. The morphological questions we are now trying to answer would have been meaningless in the classical period of descriptive morphology, and, in this sense, it can be submitted that progress is being made.

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BIOCHEMICAL DIFFERENTIATION DURING AMPHIBIAN DEVELOPMENT*

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Studies on the Primary Organizer

BIOCHEMICAL studies of the so-called primary organizer or neural inductor of the amphibian embryo have been directed along two main lines. In the first place, following the discovery that induction of the nervous system could be brought about by agencies other than living embryonic tissues, attempts were made to determine, by various implantation techniques, the nature of the chemical substance or substances which presumably were involved in the process. The literature on this subject is not without interest, but, it must be confessed, our knowledge of the chemistry of the natural inductors has not been greatly enhanced by the numerous studies of this type. Nevertheless, it can hardly be doubted that the interaction between the invaginated chorda mesoderm and the presumptive neural plate, leading to the histogenetic and morphogenetic differentiation of the nervous system, involves the operation of specific chemical substances even though their precise nature and the means through which they exert their influence are unknown. Moreover, it seems increasingly clear, especially in view of Holtfreter's (1944, 1945) recent results, that embryonic tissue contains inducing substances in bound or inactive form and that, as suggested by Needham (1942), a number of physical and chemical agents are effective in bringing about their release.

Respiratory Rates of Dorsal Lip and Ventral Ectoderm. The second line of work has been concerned with an analysis of the metabolic characteristics of inducing and non-inducing parts of the embryo in an attempt to determine whether the developmental events associated with the phenomenon of induction could be correlated in any way with special biochemical processes or properties in particular regions of the embryo. It is perhaps natural that some of the earliest efforts in this direction should concern the study of the overall metabolism of various regions of the gastrula as revealed by their total oxygen uptake. The first investigation was undertaken by Brachet (1935), when he measured the effects of localized cautery on the respiratory metabolism of *Rana temporaria* gastrulae. His results indicated that destruction of the cells in the vicinity of the dorsal lip produced a somewhat greater reduction of oxygen consumption than when a corresponding area from the opposite side of the embryo was removed. Measurements of the carbon dioxide production of dorsal lip and ventral ectoderm explants yielded essentially similar results (Brachet, 1935). In these studies, quantitative estimations of the amount of tissue used in the comparative tests were not made, so that the results, although highly suggestive, had to be interpreted cautiously. However, in later work (Brachet, 1936), Kjeldahl nitrogen determinations were made on the explants of *Discoglossus* gastrulae after their carbon dioxide production had been measured, and the results indicated in this case that the rate of carbon dioxide production by pieces of gastrula from the dorsal lip region was unquestionably greater than that of ventral ectoderm.

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At the same time, the oxygen consumption of explants of *Triton alpestris* gastrulae was measured by Waddington, Needham, and Brachet (1936) in a modified Gerard-Hartline respirometer. In most cases, the dorsal lip was found to have a greater oxygen consumption than the ventral ectoderm, but when respiration was related to dry weight no difference between dorsal lip and ventral ectoderm was noted.

By an ingenious method, Brachet and Shapiro (1937) measured simultaneously the oxygen consumption of the two halves of the intact gastrula of *Rana sylvatica*. The egg was placed in the center of a capillary, so that the respiration of one hemisphere could be directly compared with that of the other. When the gastrula was oriented so that its dorsal-ventral axis was perpendicular to the long axis of the capillary, no difference in the respiratory rates of the two halves of the egg was observed. However, when the dorsal lip region and ventral ectoderm were directed toward opposite ends of the capillary, the respiration of the hemisphere containing the dorsal lip was some 40 per cent greater than that of the opposite side. This does not necessarily indicate that the dorsal lip has a more intense metabolism than the opposite side of the gastrula, for, as Fischer and Hartwig (1938) have pointed out, it is unlikely that the index drops in the two limbs of the capillary were being influenced by equal amounts of respiring tissue.

Following this, Brachet (1939) further investigated the respiration of explants of gastrulae, using large numbers of pieces in the Meyerhof-Schmitt respirometer. Because of the insensitivity of the instrument, respiration had to be followed for 20 to 30 hours. Brachet's results indicated that the respiration of the dorsal lip region of *Discoglossus* gastrulae was, on the average, some 30 per cent greater than that of the ventral ectoderm. Carbon dioxide production averaged 84 per cent greater in the dorsal lip region. In a second series of experiments, Brachet (1939), using the Brachet-Shapiro apparatus for measurements on the intact gastrula, reported that the average value for the respiration of the dorsal lip-containing half of the gastrula of *Rana temporaria* was not significantly different from that of the half containing the ventral ectoderm. However, carbon dioxide production, measured on the intact gastrula by a modification of the same method, was again seen to be much greater in the half containing the dorsal lip.

Fischer and Hartwig (1938), using as many as 60 explants in Warburg respirometers in experiments lasting from 16 to 23 hours, obtained an average figure for the respiration of the dorsal lip of 2.34 cu.mm. per 10 mg. dry weight per hour. The corresponding value for the ventral ectoderm was 1.83. In experiments of shorter duration, *i.e.*, 6 to 9 hours, the difference was less, the averages being respectively 2.13 and 1.92.

Using the Cartesian diver technique, Boell and Needham (1938, 1939) and Boell, Koch, and Needham (1939) measured the oxygen consumption of explants of dorsal lip and ventral ectoderm of two species of amphibia. In these experiments, made on single explants, the respiratory measurements were confined to a period of 3 hours following isolation of the explants and their insertion into the divers. These investigations showed no significant difference in the average respiratory rates of dorsal lip and ventral ectoderm, and confirmation of this result was obtained with *Rana temporaria* by Needham, Rogers, and Shen (1939). In our work, some variation was noted in the relative rates of respiration of the two kinds of tissue from experiment to experiment. Out of a total of 28 tests in which the tissue fragments whose respiration was being compared were isolated from the same gastrula, 9 showed the rate of oxygen uptake of the dorsal lip to be greater than that of ventral ectoderm. In 16 experiments the opposite was true, and in 3 cases the respiratory rates of the two regions were identical. Brachet (1945, p. 389) has suggested that the variation in our results may have been due to the effects of tissue cytolysis at the air-fluid interface because of the small amount (2 cu.mm.) of Holtfreter's solution used in the divers. However, calculation shows that, even with the largest tissue samples, the volume of saline in which the tissue was supported was about 8 times that of the tissue, and in the majority of the experiments this figure was nearer 20. Examination of the tissues in the divers at the end of the experiments revealed that they remained well-healed and could be removed intact for Kjeldahl determinations. Furthermore, in most cases the respiratory rate was constant during the experimental period. "*Ce serait*," states Brachet, "*un indice d'un état physiologique demeuré normal.*" As will appear below, it is highly probable that the variation in our results was due to the fact that the dorsal lip and ventral ectoderm differed in composition from experiment to experiment.

The experiments which have been mentioned in the discussion thus far are summarized in TABLE 1. They were performed on embryonic material from different species and under various experimental conditions. It is unlikely, however, that such factors are solely responsible for the lack of agreement in the results. Brachet (1939) has stated that, in experiments of short duration, the respiratory rates of dorsal lip and ventral ectoderm are essentially identical, but that in longer experiments the respiratory rate of the dorsal lip is greater. This is due, he believes,

TABLE 1

SUMMARY OF WORK ON THE RESPIRATORY RATES OF DORSAL LIP AND VENTRAL ECTODERM IN THE AMPHIBIAN GASTRULA

Investigator	*Material*	*Rate DL*	*Rate VE*	*DL/VE*
Waddington, Needham, & Brachet	*T. alpestris*[1]	0.23	0.21	1.10
Brachet & Shapiro	*R. sylvatica*[2]	85	58	1.47
Fischer & Hartwig	*A. mexicanum*[3]	2.34	1.83	1.28
Fischer & Hartwig	*A. mexicanum*[3]	2.13	1.92	1.11
Boell & Needham	*Discoglossus*[4]	4.80	4.93	0.98
Boell & Needham	*A. mexicanum*[4]	3.21	3.18	1.01
Boell, Koch, & Needham	*A. mexicanum*[4]	5.3	4.2	1.26
Brachet	*R. temporaria*[5]	0.164	0.153	1.07
Brachet	*Discoglossus*[4]	4.1	3.1	1.31
Needham, Rogers, & Shen	*R. temporaria*[4]	3.74	3.78	0.99

Respiratory rates (µl. O_2 consumed per hour) are based on the following units of tissue: *1.* milligram dry weight; *2.* gram wet weight; *3.* 10 milligrams dry weight; *4.* milligram nitrogen; *5.* not stated by the author.

to the fact that the dorsal lip tissue differentiates more completely after excision from the embryo than ventral ectoderm and that its respiratory rate therefore increases during the process, whereas that of ventral ectoderm does not. Although this seems a plausible explanation, it is likely that the variations in the results reported above are due even more to fundamental differences in technique. Perhaps the factor of greatest importance has been the failure of various workers to use corresponding areas of dorsal lip and ventral ectoderm for comparison. This is understandable, however, when it is remembered that the various presumptive areas of the gastrula have only statistical boundaries. So-called dorsal lip material varies in composition with the age of the gastrula. In the very youngest stages, it consists exclusively of chorda mesoderm, most of which has not yet been invaginated. In later stages, it is composed of a double layer of tissue, the outer one representing presumptive neural plate and the inner layer comprising chorda mesoderm or archenteron roof. In a given isolate of dorsal lip, the relative amounts of mesoderm and ectoderm will vary, accordingly, with the age of the gastrula from which it is taken as well as with the size of the explant. Furthermore, the term "ventral ectoderm" denotes no well-defined zone in the gastrula, and the respiration of a fragment of ectoderm may therefore be expected to vary somewhat, depending upon its position in the gastrula.

Regional Variation in Respiration of Gastrular Explants. In more recent work, some of these considerations have been taken into account, and comparisons have been made of the respiratory rates of presumptive regions taken from various levels of the gastrula (Boell and Nicholas, 1940; Boell, 1942; Barth, 1939, 1942). The results of these experiments are summarized in TABLE 2 and indicate that differences of considerable magnitude appear in the oxygen consumption of different areas from the same gastrula. There seems to be a gradient of respiratory activity

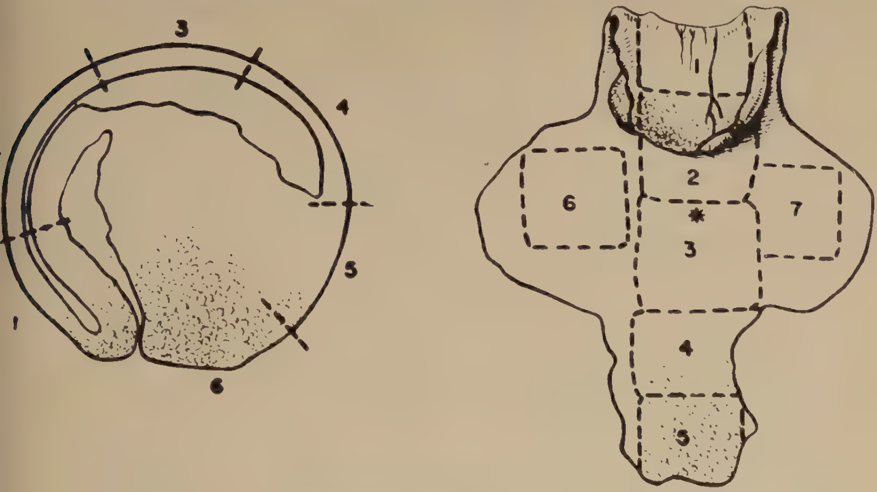

FIGURE 1. Diagram showing method of dissecting pieces of tissue used in the experiments summarized in FIGURE 2 and TABLE 2. Piece 1 refers to the dorsal lip region.

extending from the animal pole to the vegetal pole with the more anterior part of the presumptive neural plate showing the highest rate of respiration. As might be expected, some variation in the absolute levels of the various regions is apparent from gastrula to gastrula, but the general pattern of respiratory rate is similar to that indicated in FIGURE 2.

TABLE 2

RESPIRATION OF VARIOUS REGIONS OF THE GASTRULA

Region	*No. expts.*	Q'_{O_2}	*P E ave.*
Dorsal lip	31	2.1	0.07
Presumptive neural plate	14	4.9	0.28
Anterior ectoderm	20	4.5	0.16
Posterior ventral ectoderm	10	3.0	0.19
Yolk endoderm	13	1.3	0.09
Right lateral ectoderm	3	3.1	0.14
Left lateral ectoderm	6	3.4	0.31
Chorda mesoderm	6	1.2	0.09

Furthermore, it appears that the differences in respiration among the various regions are more marked when the pieces used for comparison are small and represent well-localized areas. The use of small pieces of tissue in respiration studies has been criticized by Child (1946, p. 127). It may be stated, however, that the pieces of tissue used in the work summarized in TABLE 2 were somewhat larger than those used routinely in transplantation experiments.

Barth (1942) has measured the oxygen uptake of explants of three species of Amphibia by means of a micro-Winkler technique. Although his measurements were made on several gastrular pieces of somewhat larger size than those in our experiments, the results obtained were similar to those already mentioned. It thus appears that the relative respiratory rate of dorsal lip tissue depends upon the particular region

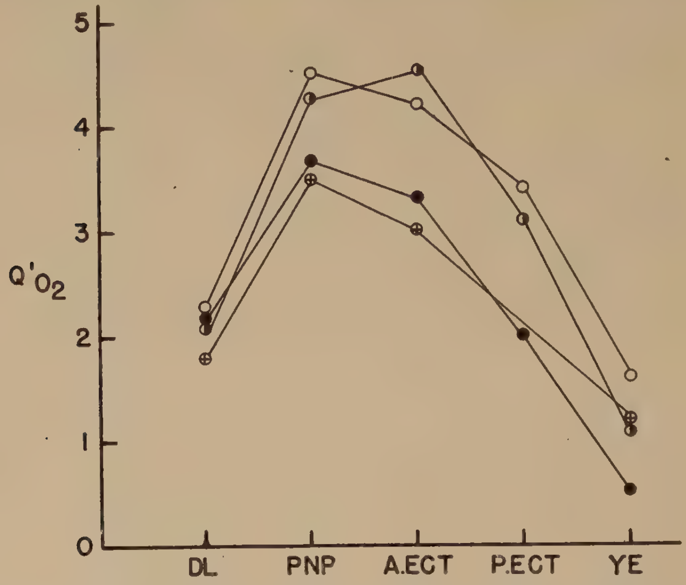

FIGURE 2. Graph showing the variation in the respiratory rates, Q'_{O_2}, of various regions of the gastrula of *Amblystoma punctatum*. DL=dorsal lip material. PNP=presumptive neural plate. A.ECT=ectoderm from the region of the animal pole. P.ECT=ectoderm from region between animal and vegetal pole on ventral side of the gastrula. YE=yolk endoderm.

of the gastrula with which it is compared, being higher than that of ectoderm if the latter tissue is removed from near the vegetal pole, and lower if it is taken from the animal pole of the embryo. This factor is doubtless mainly responsible for the differences in the results of various workers reported in TABLE 1. Moreover, it seems the most likely explanation of the variation in the relative respiratory rates of dorsal lip and ventral ectoderm from gastrula to gastrula reported by Boell and Needham (1939).

Dorsal lip material represents a double layer of tissue while the other regions selected for study are generally thinner. One might question, therefore, whether sufficient oxygen from the air in the gas space of the divers was diffusing into the dorsal lip tissue to maintain respiration at its potential maximum. Therefore, a number of experiments were performed in which the divers were filled with oxygen instead of air and data obtained on the relative respiratory rates of the various regions of the gastrula under these conditions. Comparative figures are presented in TABLE 3. These experiments show clearly that the difference between dorsal lip and ectoderm is due to intrinsic dissimilarities in the tissues rather than to differences in the availability of oxygen. The lower respir-

TABLE 3

COMPARISON OF THE RESPIRATORY RATES OF REGIONS OF THE GASTRULA IN AIR AND IN PURE OXYGEN

Region	*Air*		*Oxygen*	
	Q'_{O_2}	*Per cent*	Q'_{O_2}	*Per cent*
Dorsal lip	2.1	43	1.6	50
P. neural plate	4.9	100	3.2	100
Ant. ectoderm	4.5	92	3.2	100
Post. ectoderm	3.0	57	1.8	56
Yolk endoderm	1.3	27	0.8	25

atory rates in oxygen as compared with the average values in air are simply a reflection of the variation in oxygen uptake in different gastrulae. Pure oxygen, apparently, has no deleterious effect on the tissue explants, and it may be recalled that Parnas and Krasinka (1921) showed that pure oxygen was without effect on the respiration of intact embryos.

Regional Distribution of Yolk and the Respiratory Rate of Yolk-Free "Active" Material in the Gastrula. The amphibian egg contains a large amount of yolk which is distributed asymmetrically between the animal and vegetal poles (Bragg, 1939; Daniel and Yarwood, 1939). Although important as raw material in developmental processes, yolk is generally believed to be relatively inert metabolically. Pickford (1943) found that the dipeptidase activity of yolk is negligible, and Boell and Shen (1944) have shown that yolk obtained by centrifuging homogenates of whole amphibian embryos possessed very little cholinesterase activity. In view of the differences in the yolk content of the various regions of the gastrula, it seemed likely that the differences in respiratory rate might simply reflect the presence of varying amounts of yolk.

Accordingly, determinations of the yolk content of pieces of the amphibian gastrula corresponding to those used in respiration experiments were made (Barth, 1942; Boell, 1942). In the latter work, pieces of gastrula were centrifuged in the Beams air-turbine centrifuge after their initial volume had been determined. Centrifugation at high speeds completely disrupted the tissues and stratified their components so that the amount of yolk could be estimated volumetrically (FIGURE 3). The yolk content of the various regions of the gastrula is summarized in TABLE 4. These figures differ rather widely from the indirect determinations of Barth (1942), but accord with the older measurements of McClendon (cited by Needham, 1931), who found that about 78 per cent of the *Rana pipiens* egg is represented by the yolk fraction.

From the data in TABLES 2 and 4, it appears that the respiratory rate of a piece of tissue is inversely proportional to its yolk content or directly proportional to the so-called "active" material (FIGURE 4). If a direct relationship actually exists between respiration and active substance, one might reasonably expect the extrapolated curve in FIGURE 4 to intersect

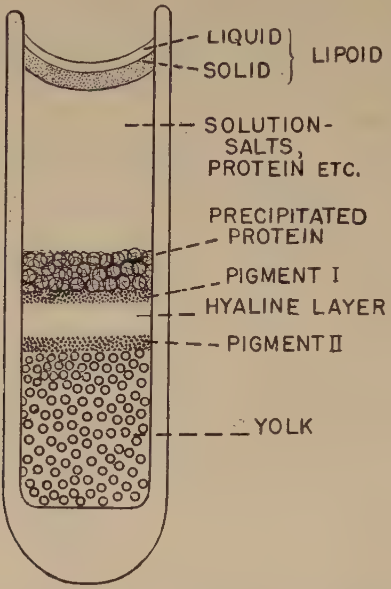

FIGURE 3. Diagrammatic representation of the stratification of gastrula explants after high-speed centrifugation.

the abscissa at zero. The fact that it does not do so presumably means that the "active" fraction still includes substances, such as lipoidal material and pigment, which are metabolically inert. It is interesting to note that, when respiration is corrected for the amount of inactive material indicated by the graph, the respiratory rates of different parts of the gastrula are approximately identical. It may be mentioned in passing that the inability to obtain, by direct means, a measure of the metabolically active fraction of a piece of tissue emphasizes again the inadequacy of the usual units such as dry weight, total nitrogen, tissue volume, etc., as quantitative indices of the amount of embryonic material used in comparative tests of this kind.

TABLE 4

YOLK CONTENT OF VARIOUS REGIONS OF THE GASTRULA AND RESPIRATION OF "ACTIVE MATERIAL" OF THE TISSUE

Region	*No. determinations*	*Per cent yolk*	*Per cent active material*	*Resp.*
Dorsal lip	7	56	44	4.8
Presumptive neural plate	7	33	67	7.3
Anterior ectoderm	7	31	69	6.5
Posterior ectoderm	7	43	57	5.2
Yolk endoderm	8	66	34	3.8
Right lateral ectoderm	2	34	66	4.7
Left lateral ectoderm	2	34	66	5.1
Chorda	2	63	37	3.3

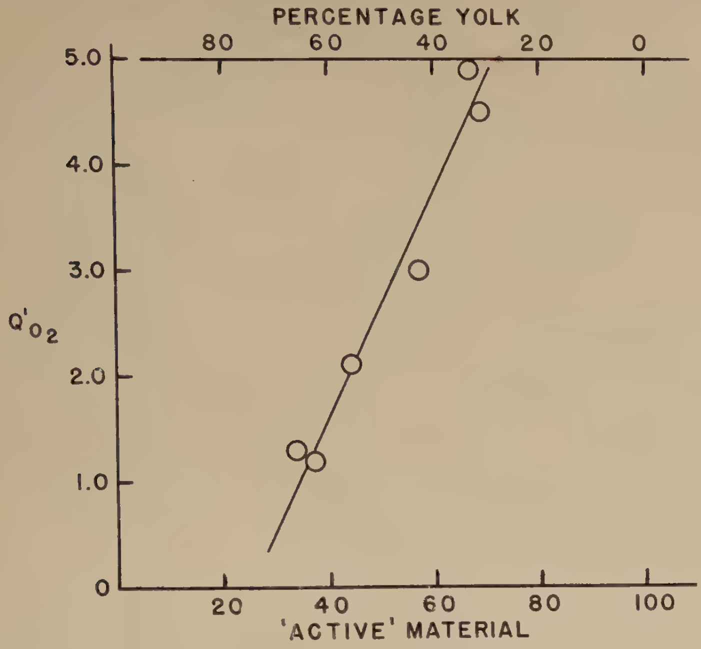

FIGURE 4. Graph showing a plot of the respiratory rate (mμl. O_2/μg. N/hour) of explants of various regions of the gastrula against the percentage yolk content of the regions.

The data summarized in TABLES 2 and 4 are of particular interest when considered in relation to Child's theory of axial gradients as it applies to organizer action. From the results of various non-quantitative tests (such as differential susceptibility to toxic substances, differential staining, etc.) which have been applied to the amphibian gastrula or to other developing or regenerating animals, Child (1929, pp. 50–51) has concluded that ". . . at the time of gastrulation the region about the dorsal lip is the region of most intense metabolic activity in the embryo and that its dominance as an organizer results from this condition." In addition, he has stated ". . . that various facts indicate a very great increase in metabolism during early stages of amphibian development up to the gastrula and that the dorsal lip region at the time of gastrulation is probably not only the region of most intense metabolism in the embryo at that stage, but also of most intense metabolism in embryonic development, because it is physiologically youngest at that stage." In other words, if these statements are interpreted correctly, Child contends that the organizer is an organizer "primarily because of its quantitatively greater activity" as compared with other parts of the embryo. More recently, the same author (1941) has again expressed this view in concluding that "in general, the natural inductors are, or at certain developmental stages, become, high gradient levels, and their inducing action apparently consists in alteration of conditions in regions representing lower levels. The inductor apparently represents a higher range of gradient levels and by raising the level of presumptive neural plate, makes possible further development." Barth (1942) has shared the opinion that "the stimulus for organization proceeds from tissues exhibiting a high rate of oxygen consumption."

Needham (1942), on the other hand, has expressed the opinion that "although gradients of susceptibility to lethal agents, intensity of vital staining, etc., had been clearly demonstrated in a large number of adult invertebrates and many embryos, no evidence whatever has been brought forward to justify a belief in the existence of 'respiratory' or 'metabolic' gradients in embryos, and very little satisfactory evidence for this in the case of adult organisms." Furthermore, after reviewing the available data on the subject, Needham concludes that there is little basis for considering that respiratory gradients are in any way connected with the phenomenon of induction in the amphibian gastrula. Child (1946), in a further attempt to support his view, has stated, however, that the data on respiratory determinations of various gastrular regions "are, at present, far from agreement with Needham," and he rejects the determinations of Boell and Needham (1939), and presumably all others which fail to show a difference in the respiratory rates of dorsal lip tissue and ventral ectoderm, as being without definite significance since no account was taken of varying amounts of yolk in the pieces used in comparative tests. "The dorsal lip cells," he writes, "contain more yolk than those of ventral ectoderm. If they respire at the same rate without correction for yolk, the metabolizing protoplasm of the dorsal lip must actually respire at a considerably higher rate than that of ventral ectoderm." It is clear, however, from an examination of the data in TABLE 4, that even after correction for yolk content, the dorsal lip does not have a higher respiration than ventral ectoderm. A more significant comparison, from the standpoint of Child's theory, would be that between dorsal lip and presumptive neural plate. However, the data in TABLE 4, as well as those of Barth (1942), provide no support for the view that these regions of the gastrula represent respectively "higher" and "lower" gradient levels in the developing system. Furthermore, it would seem unlikely that a quantitative level of metabolism, or difference of level between inducing and induced tissues, is necessary, since it has been experimentally demonstrated that induction and neural differentiation can occur in the presence of cyanide in a concentration sufficient to inhibit respiration by 80 to 90 per cent (Brachet, 1939; Barnes, 1944; Boell, unpublished experiments) and under anaerobiosis (Brachet, 1939). It is of further interest to note that Philips (1942) found no difference of significance in the respiratory rates of various presumptive regions of the early chick blastoderm. Moreover, Lindahl and Holter (1940) reported that the respiratory rates of animal and vegetal halves of sea urchin embryos (*Paracentrotus lividus*) were the same in spite of their different developmental potentialities. Thus, there can be little doubt that inductive action is not associated with quantitatively higher respiratory metabolism in the dorsal lip than in the presumptive neural plate or in other parts of the gastrula.

Respiratory Quotients and Glycolysis. Studies by different workers on the respiratory quotients of various gastrula regions are in complete agreement in showing a value of approximately unity for the dorsal lip tissue and considerably lower values for ventral ectoderm. It has already been noted that Brachet observed, in comparative tests on dorsal lip material and ventral ectoderm, a greater difference in favor of the dorsal lip for carbon dioxide output than for oxygen uptake, and this was correctly interpreted as indicating a difference in the respiratory quotients of the two regions. Subsequent work, summarized in TABLE 5, has abundantly confirmed Brachet's initial observation.

TABLE 5

SUMMARY OF RESPIRATORY QUOTIENTS OF DORSAL LIP AND VENTRAL ECTODERM FROM THE AMPHIBIAN GASTRULA

Investigator	*Material*	*R.Q. DL*	*R.Q. VE*
Brachet	*Discoglossus*	1.02	0.73
Brachet	*R. fusca*	0.97	0.80
Boell, Koch, & Needham	*A. mexicanum*	0.98	0.87
Needham, Rogers, & Shen	*R. temporaria*	0.92	0.81

Brachet (1934) has shown that the R.Q. of intact amphibian eggs averages approximately 0.66 during cleavage stages but rises abruptly to approximately 1.0 during gastrulation, at which point it then remains during subsequent development. Mendes (1948, in press) has extended the study of the respiratory quotient during development of the frog, *Rana pipiens*, to include all stages from immediately after fertilization to the end of the pre-feeding period. He has found that the R.Q. of newly fertilized eggs and of embryos in the early cleavage stages is unity. However, in the late blastula stage, the R.Q. drops to an average value of about 0.7, only to rise again during gastrulation.

Boell, Needham, and Rogers (1939) have shown that the rise observed by Brachet (1934) and Mendes occurs also in isolated regions of the embryo but values of unity are reached by the dorsal lip region in advance of the ventral ectoderm. In the case of the former tissue, the respiratory quotient becomes unity as soon as gastrulation commences, suggesting that the process of invagination is in some way associated with carbohydrate metabolism. Gastrulation in the ventral tissues, presumably, is also concerned with carbohydrate breakdown and an R.Q. of 1.0, but the process appears later in development than it does on the dorsal side of the embryo and it is probable that the respiratory quotient of ventral ectoderm does not reach unity until the tissue becomes underlain by mesoderm.

These respiratory quotients, although admittedly difficult to interpret, have generally been regarded as indicating that carbohydrate metabolism predominates in the dorsal lip region and that ventral ectoderm shows a mixed metabolism. It should be recognized, however, that complete combustion of protein with ammonia as the nitrogenous end product will likewise yield a respiratory quotient near unity. However, it seems unlikely that this is the case in the dorsal lip region of the gastrula since it has been shown that very little ammonia is produced under aerobic circumstances by explants of dorsal lip (Brachet, 1939; Boell, Needham, and Rogers, 1939; Needham, Rogers, and Shen, 1939).

The conclusion which has been derived from the respiratory quotient data reported above, *viz.*, that gastrulation in some way involves carbohydrate breakdown, is made more tenable by direct determination of glycogen in various gastrular regions. Woerdemann (1933) first showed, by a histochemical method, that glycogen disappears from the dorsal lip tissue during invagination. Although the technique used in obtaining this result was not above criticism, Woerdemann's observation was confirmed by Heatley (1935) and Heatley and Lindahl (1937). These authors compared the glycogen content of corresponding regions in the blastula and gastrula of the axolotl embryo and found a significant decrease (31%) in glycogen only in the dorsal lip region (see TABLE 6).

TABLE 6

TOTAL GLYCOGEN IN THE AXOLOTL EMBRYO
Figures in mg. % dry weight
(From HEATLEY & LINDAHL, 1937)

Region	*Blastula*	*Gastrula*	*Per cent decrease*
D 1	17.8	16.5	7
D 2	12.0	8.3	31
D 3+V 3	4.3	3.9	9
V 1	16.7	16.5	1
V 2	10.0	9.3	7

Recently, Jaeger (1945) has confirmed this finding in analyses of *Rana pipiens* embryos (TABLE 7). It is interesting that Jaeger found approximately the same percentage decrease in glycogen (33%) in the dorsal lip

TABLE 7

TOTAL GLYCOGEN IN REGIONS OF FROG EMBRYO
Figures in mg. % dry weight
(From JAEGER, 1945)

Region	*Before gastrulation*	*After gastrulation*	*Per cent decrease*
DL *in situ*	12.3	8.2	33
VL *in situ*	14.0	10.1	28
DL explant	11.2	11.1	1
VL explant	10.8	10.3	4.5
DL+ECT explant	12.8	12.5	2.5

egion as was observed by Heatley and Lindahl. Unfortunately, her nalyses did not include a comparison with other parts of the gastrula luring the same developmental period, but one may conclude, on the trength of Heatley and Lindahl's observations, that the decrease in ther regions is perhaps small. Jaeger went on to show, in confirmation f Tanaka's (1934) earlier report, that glycogen was likewise lost from he ventral lip when it underwent invagination. Apparently, glycogen oss from these regions was not an "autonomous" process, for explants of lorsal and ventral lip in saline solutions showed no appreciable decrease n glycogen. Furthermore, glycogen was not lost from presumptive dorsal r ventral lip material in hybrid embryos in which developmental arrest ccurred at the gastrula stage. Nor was glycogen loss associated with the induction of neural tissue, since no decrease of glycogen was observed in explants of dorsal lip and competent ectoderm in which neural differentiation had occurred (as indicated by histological appearance of the tissues). Glycogen loss seems not to be associated simply with the assumption of an "internal position" of the tissue after gastrulation, for Raven (1935 a and b) demonstrated that pieces of ectoderm implanted into the blastocoel of the amphibian embryo showed no decrease in their glycogen content. However, a piece of dorsal lip material implanted so as to go through its normal invaginative movements did lose glycogen. Furthermore, a piece of ectoderm implanted in the dorsal lip region and carried into the interior of the embryo by the invagination of the dorsal lip lost glycogen in the same way as did the dorsal lip. From these observations, as well as from those of Jaeger, it appears highly probable that glycogen disappearance is in some way connected with the changes in cell shape and with cell movements during gastrulation. It may be as Needham (1942, p. 191) has suggested, that more than coincidence is involved between the contractile appearance of the blastoporal cells and their utilization of glycogen.

It is noteworthy that Jaeger's figures for dorsal lip tissue were obtained by comparing corresponding regions in embryos at stages 10 and 12, and that those for ventral lip were obtained on stages 11 and 14. Jaeger gave no indication of the time required for development between these stages, but reference to Shumway's (1940) time table for normal development in *Rana pipiens* indicates that the time interval between stages 10 and 12 is 16 hours, whereas 28 hours are required for the embryo to advance from stage 11 to stage 14. Since during these times the quantitative decrease in glycogen was about the same in both regions, the rate of glycogen loss per unit of time is correspondingly greater, indeed almost twice as great, in the dorsal lip than in the ventral lip tissue. This result is interesting in comparison with the data of Boell, Needham, and Rogers (1939) showing that anaerobic glycolysis occurred both in dorsal lip and in ventral tissues of the embryo, but that the rate of glycolysis was about three times greater in the dorsal lip than in ventral ectoderm (TABLE 8).

TABLE 8

ANAEROBIC GLYCOLYSIS ($Q_L^{N_2}$) IN DORSAL LIP AND VENTRAL ECTODERM OF THE AMPHIBIAN GASTRULA

	DL	*VE*
Rana temporaria	+0.63	+0.21
Triton alpestris	+0.42	+0.14

The Development of Enzyme Systems in the Embryo

The organic matter of the amphibian egg may be divided into two fractions. One of these is the protoplasmic or metabolically active portion; the other consists of storage material or yolk, and, as was indicated in the foregoing discussion, there is evidence that this fraction is relatively inert metabolically and serves mainly as a source of raw material for development. It is worthy of note that, during the period of development covering the first 600 hours after fertilization, *i.e.*, during the period of egg and larval development included in Harrison's normal stages, the embryo takes in no food. The total organic matter of the egg thus remains essentially constant; actually it decreases slightly as can be seen in data for dry weight (Dempster, 1930, 1933) or total nitrogen (Wills, 1936). However, during development, the relative proportion of protoplasmic material and yolk is constantly changing as the yolk becomes gradually transformed into new protoplasm and the products of protoplasmic activity. It would seem that the raw materials of the egg represented in the yolk are present in highly concentrated form and that their transformation into protoplasm involves considerable hydration (Gray, 1926; Dempster, 1930, 1933; Boell, 1945). Indeed the embryo of *Amblystoma punctatum* takes in, during its pre-feeding development, approximately 20 mg. of water, and the dry weight is reduced accordingly from 35 per cent at stage 18 to less than 10 per cent at stage 45.

Respiration of the Developing Amphibian Embryo. Unfortunately, we know nothing about the rate at which yolk is being transformed into protoplasm in the normal development of the amphibian embryo, since so far it has been impossible to determine the quantities of the two entities separately. However, an indirect indication of the process may be seen in the changing metabolic activity during development of the embryo as indicated by the respiratory rate (*cf.* Gray, 1927, 1929). A graphic summary of data relating oxygen consumption of *Amblystoma punctatum* to the developmental age of the embryos is seen in FIGURE 5. It is apparent, from the graph, that the amount of oxygen consumed by the embryo increases smoothly and steadily throughout development. Abrupt changes in the rate of respiration, or cyclic variations such as appear in the development of the sea urchin (Lindahl, 1939) or the grasshopper (Boell, 1935) are conspicuously absent. There is no indication of increased energy expenditure for such morphogenetic processes as gastrulation,

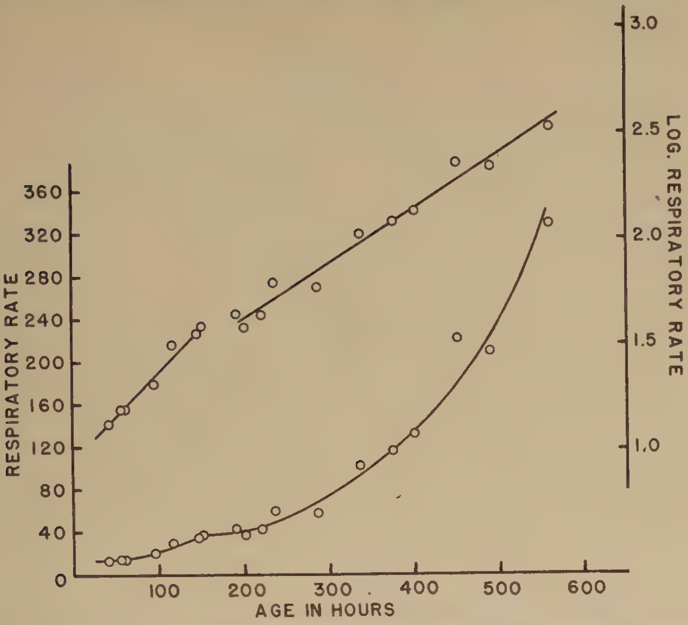

FIGURE 5. Graph showing the respiratory rate of intact embryos of *Amblystoma punctatum* during development. The arithmetic scale on the left relates to the lower curve; the logarithmic scale on the right, to the upper curve. Respiration is expressed as mμl. 0_2/100 μg. dry weight/hour.

he differentiation of the primary germ layers or their further elaboraion into the definitive tissues and organs of the embryo. Obviously, any energy expenditure for these processes—since they themselves occur gradually—is merely represented as part of the general rise in oxygen consumption which occurs during development. Similar results have been obtained for a number of other amphibian species by Wills (1936), Atlas (1938), Fischer and Hartwig (1938), Hopkins and Handford (1943), Moog (1944), Barnes (1944), Spiegelman and Steinbach (1945), and Barth (1947). It may be concluded, then, that increase in respiration is a reflection of the progressive growth in the embryo of metabolically active material.

Growth is an exponential process (*cf.* Brody, 1945, chapter 16) and obeys the general relation $X = a.e^{kt}$, or $\log X = \log a + kt \; (\log e)$. From this, it follows that any data which obey the equation should fall on a straight line when the logarithm of the growing entity is plotted against time. If respiratory increase during development is due to the growth of protoplasm, it would be expected that the course of respiration should increase exponentially. Curve B of FIGURE 5 indicates that this expectation is met. It is apparent in the curve that the rate of respiratory increase is not constant during development but occurs in two cycles with a "break" appearing on about the sixth or seventh day of development, *i.e.*, at approximately stage 32 or 34. The values of k for the two cycles of growth are, respectively, 0.0098 and 0.0060 (see TABLE 9).

TABLE 9

TABLE OF CONSTANTS FOR THE GROWTH OF VARIOUS ENZYMES AND BIOCHEMICAL ENTITIES IN THE EMBRYOS OF *AMBLYSTOMA PUNCTATUM*

	k
Respiration—40 to 180 hours	0.0098
Respiration—180 to 560 hours	0.0060
Cytochrome oxidase*—60 to 560 hours	0.0075
Cytochrome oxidase†—115 to 630 hours	0.0075
Succinic oxidase—115 to 630 hours	0.0061
Cholinesterase—200 to 400 hours	0.0196
Cholinesterase—400 to 600 hours	0.0092
Volume of central nervous system—200 to 560 hours	0.0006

* *p*-Phenylenediamine used as substrate. Determinations in Warburg apparatus.
† Ascorbic acid used as substrate. Determinations in Cartesian diver apparatus.

Atlas (1938), Moog (1944), Barnes (1944), and Spiegelman and Steinbach (1945) have reported a similar situation in the respiration of *Rana pipiens* and *Rana sylvatica*, but in these species the break occurs after the end of gastrulation, that is, much earlier than in the salamander embryo. A semilogarithmic plot of Barth's (1947) data for *Rana pipiens* similarly shows a break at about stage 15 (Shumway's table). The older data of Bialascewicz and Bledovski (1915, data taken from Needham, 1931, p. 677) likewise can be expressed by the equation mentioned above. In this work, the inflection in rate occurs at about the time when the external gills make their appearance, *i.e.*, at about 100 hours of development.

A semilogarithmic plot of the data for *Amblystoma tigrinum* (Hopkins and Handford, 1943) and *Amblystoma mexicanum* (Fischer and Hartwig, 1938) shows a break at about the same stage as in *Amblystoma punctatum.* Thus, it appears that a difference in the appearance of the break is characteristic of Urodeles and Anurans. It would be of interest to investigate this point further. It may be mentioned, in passing, that a similar inflection in the respiratory rate of the chick embryo can be seen when the data of Murray and Hasselbalch (Needham, 1931, Figure 156, p. 717) and Romanoff (1941) are plotted on a semilogarithmic scale. In the chick, the change in rate occurs at about the seventh day of incubation.

The fundamental cause of the break in the respiratory curves for these forms remains obscure, although it can hardly be doubted that the changing slopes of the curves reflect in some way the rate of transformation of yolk into active protoplasm. It is of considerable interest that in *Amblystoma* the change in rate of respiratory increase coincides with the time when the embryo begins to deviate markedly from the spherical form, and occurs only slightly before the initiation of the heartbeat and the establishment of circulation. Perhaps the rate of respiratory increase is limited to some extent by the development of respiratory surface as well as by the maturation of the circulatory system as an oxygen carrier and as a means of transporting raw materials from the yolk mass to the rest of the embryo.

Cytochrome Oxidase. It has been shown by a number of investigators that the amphibian egg is highly sensitive to cyanide (Brachet, 1934; Barnes, 1944; Boell, 1945), and it has been generally concluded that respiration in the embryos of Amphibia proceeds by way of the cytochrome-cytochrome oxidase system. Measurements of the cytochrome oxidase activity of *Amblystoma punctatum* embryos (Boell, 1945) show that there is sufficient activity of this enzyme during all stages of development to account for the respiration of the embryos. Figure 6, curve *A*, illustrates that the increase in cytochrome oxidase activity during development follows a course similar to that of respiration, and like respiration increases exponentially with time. The similarity in the shapes of these curves, as well as the marked sensitivity to cyanide, thus suggest strongly that respiration is largely mediated by the cytochrome-cytochrome oxidase system during development. However, the concentration of cytochrome oxidase apparently is not the factor which limits the rate of increase in respiration, since k for growth of cytochrome oxidase is 0.0075 while for respiration k is 0.0060.

Spiegelman and Steinbach (1945) have reached a similar conclusion from a study of cytochrome oxidase in *Rana pipiens* embryos. These authors report that, although increase in respiratory rate occurs, cytochrome oxidase activity remains uniformly high throughout development and that enzyme synthesis apparently does not take place. This result is somewhat surprising, for it suggests that the unfertilized egg contains the full complement of cytochrome oxidase necessary for the embryo throughout development and that during the process the enzyme merely becomes distributed to the various cells and tissues of the growing organism. Furthermore, synthesis of the enzyme has been demonstrated in the embryos of other forms, as, for example, the grasshopper (Bodine and Boell, 1935; Allen, 1940) and the chick (Albaum and Worley, 1942; Albaum, Novikoff, and Ogur, 1946). Moreover, it may be mentioned that Spiegelman and Steinbach made measurements on embryos only up to stage 19 of Shumway's (1940) table which corresponds approximately with Harrison's stage 34 or 35 for *Amblystoma punctatum.* In *Amblystoma*, the major increase in enzyme activity occurs after this stage, and unpublished experiments of the author indicate that an increase in cytochrome oxidase occurs during the comparable period of development in *Rana pipiens* as well. These experiments show, in addition, that determinations of cytochrome oxidase in early stages of *Rana pipiens* are complicated by the fact that reducing substances in the egg homogenates apparently interfere with the oxidation of *p*-phenylenediamine (compare Brachet, 1945, p. 350), and the full activity of the enzyme may not be obtained by the usual methods. It is interesting to note that Brachet's (1934) paper shows that *p*-phenylenediamine actually decreased slightly the oxygen consumption of homogenates of *Rana temporaria* eggs.

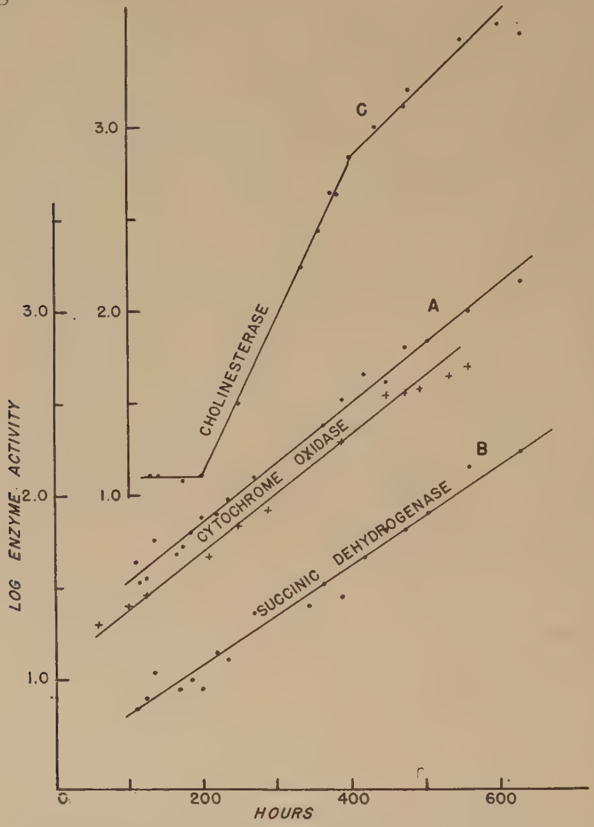

FIGURE 6. Semilogarithmic plot of enzyme activity against age of embryos of *Amblystoma punctatum*. The right-hand ordinate relates to cholinesterase activity, the curve for which has been shifted vertically to avoid superposition of curves. Enzyme activity is expressed as mμl. gas produced or consumed in the Cartesian diver apparatus/100 μg. dry weight/hour.

Succinic Oxidase. FIGURE 6, curve *B*, likewise shows that synthesis of succinic oxidase, as measured by the method of Schneider and Potter (1943), occurs throughout development (Boell, 1946a). Between 100 hours after fertilization and the end of the pre-feeding period (stage 46), succinic oxidase activity increases exponentially with time, the growth constant, *k*, being 0.0061. This is significantly less than the constant for cytochrome oxidase, but practically identical with that of the respiration of intact embryos during the same period of development. It is of interest that a break, comparable to that for respiration, is not apparent in the growth curves for either of these enzymes.

Schneider and Potter (1943) assume that measurement of the succinic oxidase system by their method actually determines the activity of succinic dehydrogenase. It is fairly certain that the limiting factor in the overall rate of oxygen uptake is neither cytochrome oxidase nor cytochrome, since the former is usually present in excess in the embryo homogenates and the latter can be supplied in excess by the addition of cytochrome *c*, but the method is incapable of distinguishing whether succinic dehydrogenase or some intermediary between succinic dehydrogenase and cytochrome *c* is the limiting factor in the reaction. It will be interesting to have data on succinic dehydrogenase activity alone by the ferricyanide method of Quastel and Wheatley (1938), in order to gain information on this point. When such data are at hand, it may be possible to assess the significance of the identity in growth rates of respiratory and succinic oxidase activities.

Cholinesterase. So far, in this discussion, evidence has been presented in support of the view that increase in respiratory and enzyme activity during development is a reflection, and to some extent a measure, of the production of metabolically active protein from the storage proteins and other materials in yolk. The question naturally arises as to whether the growth of all enzymes may be expected to follow a similar course or whether there is evidence of specialization and individuality in these processes of biochemical differentiation. The results of a study of the development of cholinesterase activity in the embryo of *Amblystoma punctatum* provides interesting information along this line. FIGURE 7, taken from Sawyer's (1943) paper, shows that cholinesterase activity increases progressively throughout the major part of the developmental process. In premotile stages, the increase in cholinesterase activity is slight, but at the time of beginning motility in the embryos it increases abruptly. From a correlation of the development of behavior according to the pattern described by Coghill (1929) and the development of cholinesterase activity, Sawyer was led to conclude that functional maturation of the neuromuscular apparatus of *Amblystoma* coincides with the development of cholinesterase to a quantitatively high level.

The shape of the curve in FIGURE 7 is similar, in some respects, to the curves relating respiration, cytochrome oxidase, and succinic oxidase to time of development. However, closer examination of these curves reveals that they are only superficially similar. We have repeated Sawyer's work in assaying the cholinesterase activity of embryos during development and have fully confirmed his observations. As curve *C* of FIGURE 6 shows, there is essentially no increase in cholinesterase activity during the period of premotile development between 130 and 200 hours (from stage 25 to stage 34). However, at the time when the embryo is first capable of making rapid, repetitive movements, cholinesterase activity is considerable and the enzyme increases in activity throughout the remainder of the period of development studied. The rate of cho-

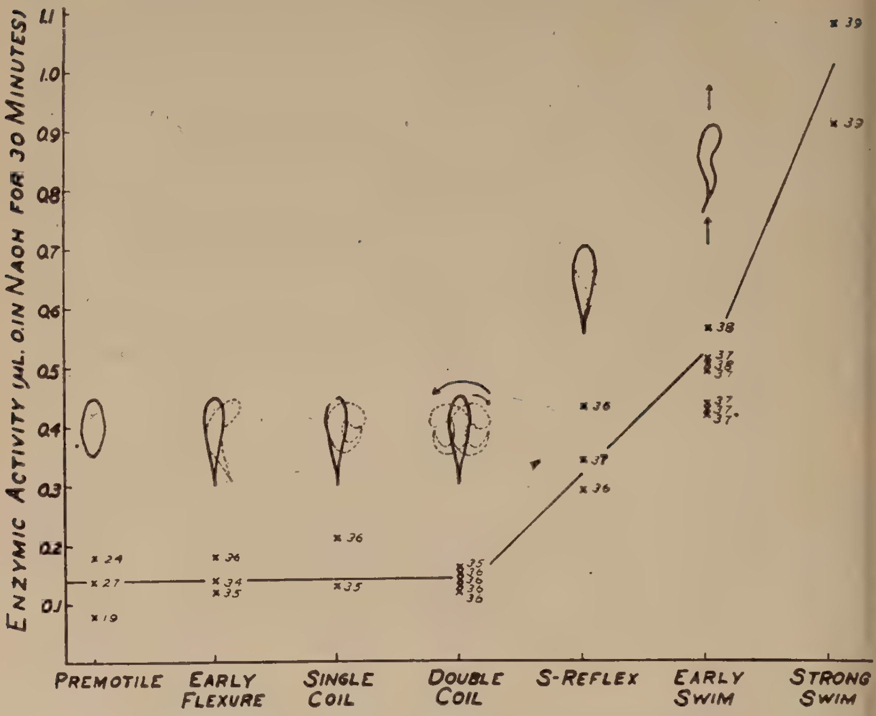

Figure 7. Summary of Sawyer's (1943) study of the relationship between the development of behavior and cholinesterase in *Amblystoma punctatum*. The numbers opposite the experimental points denote Harrison's stages.

linesterase synthesis is not constant, however, throughout the entire period. A break in the growth curve occurs after 400 hours or at approximately stage 41–42. Before the inflection, k is 0.0196, afterwards it is 0.0092. It is of considerable interest to note that the time at which the break occurs is correlated with decreased responsiveness of the embryo to mechanical stimulation, as shown in Detwiler's (1946 a and b) studies.

It would appear, from the differences in their growth rates, that the various processes of biochemical differentiation described in the foregoing discussion have certain specific and individual characteristics. If it be true that the increase in activity of respiration and respiratory enzymes reflects an increase in metabolically active material, it seems equally true that the changes in cholinesterase activity do not. The measurements of cholinesterase activity mentioned above were made on homogenates of whole embryos, but there is evidence that the enzyme is associated, at least in early stages, with the development of the nervous system. Sawyer (1943) and Boell and Shen (1944), in comparative studies of the enzyme activity of neural and non-neural tissues, were able to demonstrate a marked localization of the enzyme in the nervous system as early as stage 19 (closing neural folds), and that the difference between neural and non-neural tissues became progressively greater during subsequent development. Furthermore, assays of cholinesterase activity in secondary nervous systems, produced experimentally by the inductive action of dorsal lip tissue (chorda mesoderm) which was implanted into the blastocoele of host embryos, gave unmistakable evidence of an increased concentration of cholinesterase in the differentiating nervous system (TABLE 10; Boell and Shen, 1944). In this study, the results indicated that the cholinesterase values for the secondarily induced nervous system were quantitatively of the same order as those of the primary nervous system of the embryo and considerably higher than those of ectoderm or skin. Thus, these experiments indicate that the phenomenon of induction alters the biochemical as well as the morphological fate of the induced tissue.

TABLE 10

CHOLINESTERASE IN EXTRACTS OF PRIMARY AND SECONDARY NEURAL TISSUES
(From BOELL & SHEN, 1944)

	Q'_{ACH}		
Stage	*Primary nervous system*	*Secondary nervous system*	*Host ectoderm*
26	+32	+38	+22
34	+45	+17	+ 6
34	+66	+58	+14

Q'_{ACH} = mμl. CO_2 produced in hydrolysis of acetylcholine by extract containing 1 μg. N per hour.

During development, the nervous system increases in size, and one is therefore led to inquire whether the increase in cholinesterase activity in the embryo may be simply due to the growth of the nervous system in volume. It is obviously impossible to obtain a measure of all the neural tissues in the embryo, because of the development of nerves and other peripheral elements, but it was thought that some information on this point could be gained by making a study of the growth in volume of the central nervous system. The data were secured by projecting 10 μ serial sections of *Amblystoma* embryos at a magnification of 150 times. The outlines of the central nervous system were drawn on paper and the areas of the tracings determined by means of a planimeter. In the head and trunk region, each section was drawn and analyzed, but in the tail, where the diameter of the spinal cord was fairly uniform over relatively long distances, only every fourth or eighth section was measured. The sum of the planimetric readings was then taken as a measure of the volume of the central nervous system at each stage. Although the shrinking of nervous tissue in fixative may not be uniform at all stages of development, it was felt, nevertheless, that this method of determining the volume of the nervous system in the embryo yields data which are infinitely more accurate than any which could be obtained from dissected and weighed organs. FIGURE 8 indicates that the growth of the nervous system is essentially uniform between stages 34 and 46 and occurs at a relatively low rate; indeed, the rate of growth is only one-thirtieth that of cholinesterase. In addition, inspection of the curves shows that they are qualitatively quite different. It thus appears that the increase in the concentration of cholinesterase is not simply a function of the growth in volume of the central nervous system. No doubt, the increase in enzyme activity parallels, to some extent, morphological and physiological differentiation of the nervous system. However, part of the increase of enzyme content of the whole embryo is unquestionably due to the development of cholinesterase in non-neural tissues of the embryo, for example, in muscle (Sawyer, 1943).

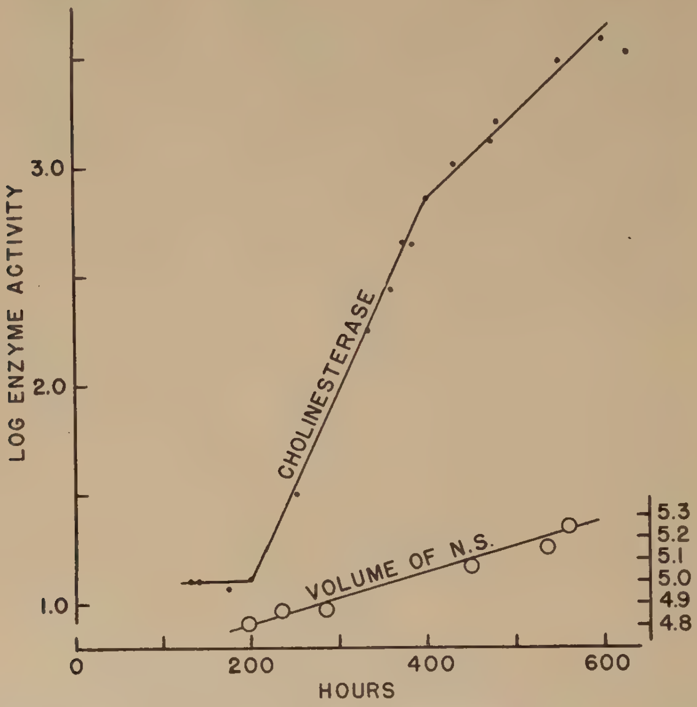

FIGURE 8. Curves showing the relative growth rates of cholinesterase activity and the volume of the central nervous system in *Amblystoma punctatum*. The upper curve is the same as curve *C* in FIGURE 6.

The Effect of Di-isopropyl Fluorophosphate. Di-isopropyl fluorophosphate (DFP) is one of a group of alkyl fluorophosphates studied by the chemical warfare services of both this country and England during the last war. According to Dixon and Needham (1946), DFP was found to inhibit cholinesterase and other esterases but had no effect on the activity of a number of other enzymes. The compound is characterized by its ntense anticholinesterasic activity, a concentration as low as 10^{-11}M roducing a definite effect. Dixon and Needham conclude that the comounds to which DFP belongs "are the most powerful and specific enyme inhibitors known." Mazur and Bodansky (1945) reported that the nhibition produced by DFP was irreversible as judged by the inability f serum esterase to regain its activity after prolonged dialysis (however, ee Bulloch, Grundfest, Nachmansohn, Rothenberg, and Sterling, 1946). They also showed that regeneration of cholinesterase activity in the nervous systems of experimental animals treated with DFP required an extremely long time. In rabbit brain, for example, cholinesterase activity was only 90 per cent of normal 50 days after treatment.

In view of these properties of the compound, it was felt that treatment of *Amblystoma* embryos with DFP so as to produce what has been aptly termed a "biochemical lesion" might yield interesting information on the functional importance of cholinesterase (Boell, 1946b). It may be recalled that Sawyer (1943) had shown that the behavior responses of *Amblystoma* embryos were depressed in the presence of the reversible inhibitor, physostigmine, and that there was a rough correlation between depression of the enzyme and the loss of normal behavioral ability.

Embryos placed in DFP in premotile stages could apparently be reared indefinitely in a concentration of 0.0001 M with no effect on morphogenesis other than slight retardation of development. However, stronger concentrations were invariably toxic and resulted in the death of the embryos in a few minutes to a few hours. In a few embryos in 0.0001 M DFP, certain abnormalities of development were noted, but in the main the DFP-treated embryos seemed to be completely normal on the basis of external appearance. Moreover, the embryos, even when reared continuously in DFP solutions for a week or more, manifested the usual behavior reactions, and, at the appropriate stages (37–38), responded to tactile stimulation with swimming movements. However, the DFP-treated embryos, although apparently normal in appearance, fatigued more readily than control animals when stimulated, and when tested by Detwiler's (1946 a and b) method for measuring behavior responses quantitatively they gave evidence of greatly reduced activity. After such embryos had been subjected to stimulation, they were usually incapable of responding again until a rest period of considerable length had elapsed. It is interesting to compare these results with the observations of Modell and Krop (1946). These investigators showed that cats that had been treated with DFP were perfectly normal in appearance and were well groomed but on stimulation gave evidence of marked myoneural dysfunction. After a few steps, the animals fell over and could not be made to move again even when given a stimulus strong enough to elicit a cry.

Embryos reared in DFP solutions and capable of responding to stimulation were found to possess cholinesterase activity of considerable magnitude, although it was much lower than in control embryos of the same developmental stage. It is of some significance to note that the concentration of cholinesterase in embryos reared in DFP was never less and usually greater than in normal animals at the time when they were first capable of responding to stimulation with rapid, repetitive movements. This is shown in TABLE 11 and would seem to indicate that a considerable excess of cholinesterase over that needed for minimal activity is present in the embryos at most stages (*cf.* Bulloch *et al.*, 1946). Under conditions of stress, the reduced cholinesterase activity may, however, lead to functional abnormality. This is clearly shown in a study of the effects of stimulation on the heart rate of the embryos. When normal embryos are stimulated until they are incapable of responding, the rate of heartbeat usually increases, but continued stimulation of DFP-treated embryos leads to a marked reduction in the rate of heartbeat. However, the beat is again normal after several minutes. Perhaps the residual cholinesterase in the tissues of the treated embryos is insufficient to remove the acetylcholine produced under such conditions of extreme activity. As a consequence, it may diffuse into the blood stream and be carried to the heart where it produces its characteristic depressing effect.

TABLE 11

CHOLINESTERASE ACTIVITY OF *Amblystoma* EMBRYOS REARED IN 0.0001 M DFP

Days in DFP	*Stage of embryo*		*Cholinesterase activity*		*Response to stimulation*
	When DFP was added	*When tested*	*Control*	*Treated*	
1	25	26	13	0	No motility
2	25	26+	13	0	No motility
—	—	37	32	—	Rapid flutter
—	—	38	63	—	Swimming response
10	22	40	436	65	Embryo swims; tires
7	27	40	436	87	Embryo swims; tires
12	20	41	973	34	Rapid flutter
14	20	42	1100	48	Behavior not noted
11	27	42+	1540	163	Embryo swims; tires
17	22	45−	2880	855	Embryo swims; tires

Apparently, synthesis of cholinesterase can take place in the embryo in the presence of DFP. TABLE 11 shows that the cholinesterase activity of embryos treated with DFP during premotile stages and reared continuously in DFP solutions increases five-to six-fold during the course of a week to ten days. After 17 days in DFP solutions, the cholinesterase activity may be as much as 65 times greater than in premotile embryos. Although the absolute level of enzyme activity is much lower in treated than in untreated animals of the same morphological stage, being on the average only 10 per cent of the control values, the rate of increase with development is approximately the same in the two groups of embryos. Apparently, therefore, development of the enzyme is not interfered with by DFP, although the newly synthesized enzyme is inhibited as soon as it is formed. The fact that the percentage enzyme activity of the reated animals in relation to the controls is approximately the same at ll stages of development, suggests that an equilibrium exists between nhibited and uninhibited enzyme.

Summary

The foregoing discussion has dealt primarily with the demonstration of regional biochemical differences in the amphibian gastrula and with the increase in activity of certain enzyme systems throughout embryonic ife. Processes of the latter type may be regarded as examples of biochemical differentiation since they represent special aspects of the synthetic or formative activities of the embryo. The discipline of chemical embryology is relatively young, and, although much progress has been made in the field in the past two or three decades, our knowledge of biochemical aspects of embryonic development is still relatively meager. It may be hoped that future work will supply us not only with more information on biochemical differences and differentiation in the embryo, but, in addition, with increased understanding of the way in which biochemical and morphogenetic processes are integrated in the complex series of events which characterize the transformation of the egg into the individual.

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FORM CHANGES DURING PRE-GASTRULAR DEVELOPMENT

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THE importance of movements of pre-gastrular materials has long been the subject of analysis by embryologists. The earliest observations on eggs have been those of studying cortical movements, movements of the materials which evidence themselves upon the surface of the egg. These movements and rearrangements are mirrored in the distribution of potencies and potentialities of the whole system in the later embryo. As is well known, Roux's (1885) early studies indicated a concept of fixity within the egg recently employed by Hamburger (1947) in an attempt to give a definite description of prelocalization at the time of fertilization or very shortly thereafter. It has been known since the time of Roux that, in certain forms, fertilization by means of an artificial insemination caused the formation of the grey crescent at a point opposite the region of sperm entrance; that the original cleavage bisects the grey crescent and coincides with the longitudinal axis of the embryo. This fixity has since been challenged and has been found not to exist in many of the other forms. It can be said with safety that there is no absolute fixed relation between the entrance of the sperm, the first cleavage, and the future axis of the embryo. While these coincidences may occur occasionally, according to statistical probabilities, they are not an interlocking determinant series.

The cortical movements of the egg have been most recently studied by Holtfreter (1944). He finds that, even in the unfertilized egg, one may have many of the cortical movements which are so characteristic of the fertilized egg. The movements of the pigment, the characteristic stranding of the pigment particles, the dispersion of pigment in certain areas —all these are obtained in unfertilized eggs. There may even be an attempt at a pseudo-gastrulation in which the surface parts of the egg, without having undergone any division at all, form lips similar to those which are found in gastrulation of a normal, fertilized type. It is true that these will not carry further, and since the material is still in a much more homogeneous condition, the materials of development are not specifically localized for further development of the organism. Holtfreter has repeated and amplified the explanations of Rhumbler (1902) in attempting to correlate the early rearrangements with the later surface movements which are so important in the allocation of materials.

Recently, there has been a renewal of the study in analyzing the movements and positions of parts in the interior of the egg. Daniel and Yarwood (1939) studied the relationship of parts in the various stages of the egg beginning with the ovarian egg, then the coelomic egg, the oviducal egg, and finally the zygote. In each of these, there is a definite stratification of the yolk. The animal pole contains smaller granules than are found in each of the succeeding fifths, until one approaches the vegetal pole where there is an increasing aggregation of the smaller particles. This is in distinct violation of our generally expressed ideas that the vegetal pole of the egg contains all the large yolk-laden elements practically devoid of pigment, and is filled with yolk platelets from which they gain the distensibility of the membrane around the yolk packets. One fails to consider that the endodermal cells at the vegetal pole of the egg are vital, tangible, and mobile elements in the picture of later development. If one surveys the figure which is given by Daniel and Yarwood (1939), it is evident that the median three-fifths of the egg are the ones which contain the heavier amounts of the yolk particles, that the upper fifth and the lower fifth, that is, the animal pole and the vegetal pole, are much less heavily laden with the yolk material. It is particularly so in the zygote stage when fertilization has taken place and the materials are beginning to separate in a much more definitive fashion than they have in the ovarian, coelomic, or oviducal egg.

Schechtman (1934, 1935, 1937), in studying ingression, was the first to notice that material could pass from the outside of the egg in the region of the vegetal pole toward the inside of the egg and, if stained in the uncleaved egg, the stains placed upon the outside to mark the regions which ingress appeared in a columnar form extending to the floor of the blastocoele. This observation is of great importance, for it shows the movement of materials from the outside to the inside of the egg. It also shows their definite alignment along a certain, particular portion of the egg. It shows, in addition, that the movement is limited to rather early stages, since in the early blastula the stain, placed as a spot on the vegetal pole of the uncleaved egg, extends from the outside to the floor of the blastocoele, but if the stain is similarly placed in the late blastula, it appears only in the lower third of the blastular floor. This observation of Schechtman is so striking that it seemed necessary to repeat the staining experiments using different degrees of stain and following the material through to a later stage of development. The phenomena of ingression are proved definitely by Schechtman's work. Its import, however, was not clearly realized by him, and since the interpretation of the significance of the cells found in the ingressed mass must remain for future study, it is necessary to repeat and amplify Schechtman's work in order to find out exactly where the cellular elements bearing the stain are forming. For this reason, the stains were applied to the outside of the egg in a manner similar to Schechtman's, beginning with the unfertilized egg and running a series similar to his. The results are quite interesting in that they show that, if an intense stain is placed upon the uncleaved egg, one can perceive as development continues that there are two lines by which the stain is sent to the inside of the embryo. The first is the one which Schechtman found and which characteristically occupies the central plane of the egg. It is as though one had a central axis of polarity in the amphibian egg due to the ingression of materials which were originally on the outside. It forms a distinct entity extending upward toward the blastocoele as a definite cone of stained material.

At the time of the late blastula, there are certain characteristic changes in organization of the interior which bring about a change in the axes of the embryo. At this time, one finds that the secondary part of the stain which had remained on the outside of the egg is carried around to the ventral lip of the blastopore, and then progresses with the ventral lip of the blastopore across the floor of the gastrocoele. The primary ingression cone has left its imprint on the center of the floor of the blastocoele and, as the gastrocoele encroaches upon the blastocoele, we find that the two colored areas approach each other as the blastocoele begins to lose its form and undergoes definitive progression toward amalgamation with the gastrocoele. In *Amblystoma punctatum*, the blastocoele remains and does not become continuous with the gastrocoele, but the stain which has come into the embryo from the ventral lip of the blastopore progresses forward in the floor of the gastrocoele until it unites with the stain which has come in through the primary ingressive mechanism.

If one applies stain to the embryo during the late blastula stage, the effect of the primary ingression with the cone of material extending inward toward the blastocoele floor is completely lost and one secures only the secondary migration of material which comes in through the ventral lip of the blastopore to the gastrocoele floor. It has been found (Nicholas, 1945) that the material which comes in through the primary ingression lies in the anterior region just under the floor of the foregut marking the region between the heart and the liver in the early stages. This is an important location and probably marks an area in which induction effects are taking place. In recapitulation, then, the staining experiments show definitely that Schechtman's primary ingression is a definite entity to which can be added, with an intensification of the stain, a secondary situation by which the stain will progress through the ventral lip of the blastopore and later join with those fragments of stain which are left from the primary ingression. Recently, Nieuwkoop (1946) has repeated Schechtman's experiments and shown the secondary relationship in an exceedingly clear way. Nieuwkoop interprets the secondary portion of the ingression which comes in through the ventral lip of the blastopore as lying in the surface of the midgut. The primary ingression coincides with the material which Schechtman found in *Triturus* and Nicholas in *Amblystoma.*

It is clear that polar orientation is influenced by the early cortical movements which, in themselves, are a reflection of the reorientation of the materials within the egg. Polar orientation has been said to occur in the ovary (Child, 1941), and undoubtedly in the amphibian egg this

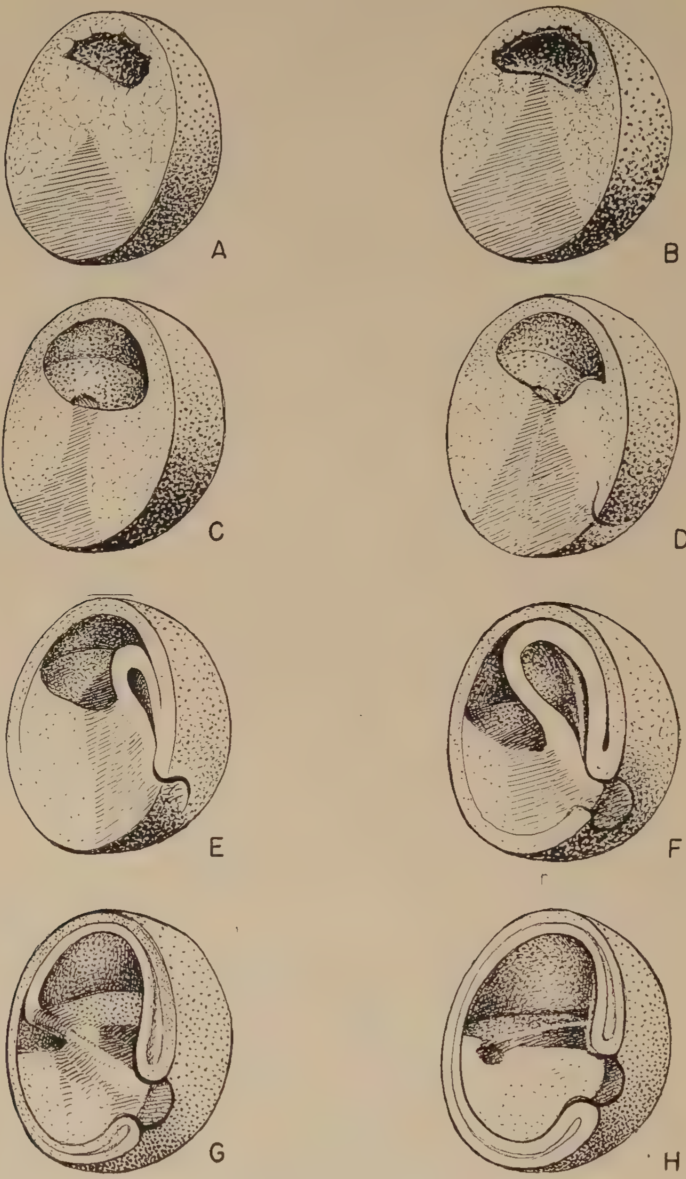

FIGURE 1. (*For description see facing page*)

is partially correct. There is, however, a marked deviation in the animal-vegetal axis shortly before fertilization and continuing afterwards. This may result in the marking of the axis 30 degrees from the original line at the time that the egg was laid. This reaction has been neglected to a large extent because we have not observed the difference in the polarity of the egg before fertilization and afterwards, but if one watches the egg one can see the change in the polarity of the animal-vegetal axis shortly after fertilization and to some extent before then.

The ingressive phenomena are essentially those which lead to the orientation of the mass within the egg after the early fertilization change in the polar axis. The inertness of the egg material which had formerly been thought to be burdened down by yolk has been found to be a fallacy in that an active movement of particles and materials does occur throughout the early stages before segmentation and apparently determines, to some extent at least, the relationship of the cortico-vegetal orientation.

The converse of this type of reaction is seen in the capacity of the embryo to undergo a tremendous amount of development without any of its yolk taking part in the reaction. This is true if one takes into account the fact that the yolk is undergoing a development of its own and has been in contact with the ectodermal and mesodermal portions of the system during their development. Nieuwkoop (1946) removed all of the yolk in the early neurula stage of amphibian embryos. When all of the yolk mass is removed from the neurula, it leaves the mesoderm which has moved inside through the blastopore, the entire chorda mesoderm which has underlain the nervous system, and the lateral mesoderm in its normal position. All of these migrations have been completed before the stage of the neurula. When the yolk is removed in this way, an embryo develops which is deficient in the head region, lacks gills, stomodaeum, all of the intestinal tract and its derivatives, and also the heart. Most of the nervous system, the lateral body wall and musculature are intact, but there is no splanchnopleure whatsoever. That is, the material which was destined to form smooth musculature of the gut is not formed. This speaks for a strong dependence of smooth musculature formation upon the gut structures which it normally surrounds. Another interesting phenomenon occurring after this operation is that two full sets

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FIGURE 1. A (stage 5). The stain placed upon the vegetal region of the uncleaved egg extends upward in a cone, which in section appears as a wedge, with its apex projecting into the upper half of the blastula. In B (stage 7), the stain has reached the floor of the blastocoele, and in C is beginning to spread over the floor surface, first as a small circle of stained material, later, stage 8, in a radiating fashion or in a complete coverage of the floor, depending upon the intensity of original staining. During the latter part of stage 8 (D), there is a tendency to have a separation of the stain into a primary segment which passes toward the blastocoele cavity and a secondary aggregation which is drawn toward the presumptive yolk plug. This reaction is more marked in E (stage 9) and F (stage 10) when the folding over of the blastocoele floor and the formation of the underside of the gastrocoele floor begin to have a common cell boundary. The two movements, the primary and secondary, are still separate in stage 11, the stain of the primary being concentrated in the walls of the blastocoele and extending through the cellular boundary into the floor of the gastrocoele (G). In H (stage 12), the two areas of stain become confluent and remain so in later stages.

or pairs of forelimbs are developed. One develops from the normal somatopleuric mesoderm and the other from what would have gone into the formation of the splanchnopleure. These two sets of limbs, two rights and two lefts, are fully formed from each of the layers, but the splanchnopleure has not formed any of the smooth muscle which ordinarily would be present. There are visible, then, two definite defects associated with muscle formation, one in the region of the heart and one in the musculature of the gut. The stomodaeum is absent, as one would expect, and also the gills. The rest of the organism, however, is remarkably regular. These results change considerably our concept of how the muscle develops if we think conventionally of smooth musculature as being primitive, the cardiac as less primitive, and the somatic striate musculature as the most advanced. The facts seem to indicate a reverse of this sequence. The striate musculature is formed from the lateral mesoderm of the marginal zone. According to the findings of Vogt (1925, 1929), the areas of somite potency are well localized at the time when the marginal zone enters through the blastopore. It is at this time that it has its later characteristics impressed upon it.

The fact that the limb could be formed from splanchnopleure was already obvious if one considers the experiments of Harrison (1925) on the reversal of the mediolateral axis of the limb. Here, the limb was turned inside out, *i.e.*, the somatopleure and splanchnopleure were reversed with reference to the yolk. The splanchnopleure gave rise to perfectly good limbs and the somatopleure undoubtedly to good smooth musculature.

Nieuwkoop (*l.c.*), when he compressed the two sides of the material from which the yolk endoderm had been removed, showed cases in which he could distinguish the formation of a coelomic cavity which, however, had no splanchnopleure in association with it. It was entirely a somatopleuric cavity. He went also one step further and stretched the embryo after depriving it of its yolk upon a collodion membrane. In this case, development occurs in a manner strikingly reminiscent of a chick blastoderm, and again shows complete deficiency of all of the splanchnopleuric parts. No heart is formed except in those embryos in which the anterior parts had secondarily come together and even here the heart is markedly deficient.

The converse of these results is shown in Bacon's (1945) experiments. Bacon removed presumptive mesoderm from the outside of the embryo before the marginal zone had passed to the interior. When this marginal zone material was placed in contact with endoderm, it formed a heart rudiment. These heart rudiments, in some cases, were remarkably regular and were formed only after contact with the endoderm. There was no attempt to have the endoderm stay in relationship with the presumptive mesoderm, but they were dissociated after a period of contact, and yet remarkably normal hearts were secured, these hearts being kept entirely in isolation conditions. The fact that the tissue which ordinarily would have developed into normal striate muscle forms a heart after having been placed in contact with endoderm shows definite inductive action of the endoderm upon the marginal zone material to form heart. Since the endoderm itself was taken from the general mass of material and was not localized, it seems as though there is a generalized influence extending through the endoderm which acts upon presumptive mesoderm for heart formation. It would be interesting to check this thesis in connection with certain definite spatially specific parts of the endoderm. This, so far, has not been done. It would also be interesting to try various sections of the marginal zone to see whether they would respond in exactly the same way to the various parts of the endoderm. An interesting series of experiments could be evolved in which these two structures were tried each against the other in order to test out the inductive effects. This induction can occur at a stage earlier than nervous system induction, thus placing the Spemann organizer in an entirely secondary role with regard to the formation of the embryo as compared to endodermal inductions.

Holtfreter (1944) has been studying the actions and reactions of various parts of the yolk endoderm. In many cases, he has restricted his observations to the cells which are coming in through the ventral lip of the blastopore. He finds that these cells, which form distinctive elements, are polarized and that when they are isolated in salt solution one can get at certain stages a centrifugal spreading of the non-polarized cells, and a definite polarization with an amoeboid process in the polarized cells. They have a proximo-distal axis which seems, to some extent, to be controlled in normal development by the action of associated cells upon the endodermal mass. The amoeboid process is generally extending away from the central mass of cells and Holtfreter has been able to show that, if four or five cells are placed together, they tend to have their proximo-distal axes arranged away from the center of the mass, the proximal portion being a rounded stub, while the distal part tends to be of the amoeboid type. Holtfreter likewise, in the same study, postulates that there is an independent movement in the cells of the endoderm, that this independent movement is responsible for a pulling of the structures so that they intrinsically occupy a definite mass and spread in a definite way. This, it seems, is endowing the endodermal cells with something a little more vital than one would expect, and probably more vital than the observational material would warrant. Whether the cells are pulling, or whether they have been displaced by the action of adjacent cells when acting in the normal organization of the embryo, is a question which still has to be decided. Holtfreter, however, has drawn our attention to an exceedingly important point in showing that, under conditions of isolation, the cells do certain definite things which seem to depend on mass aggregates and individual activity in response to that mass aggregate. No matter where the future of the endodermal situation will lead, these results will have a definite bearing upon our interpretation of how the floor of the gastrocoele is formed and what the effective forces are forming it. Certainly, the ventral lip of the blastopore has a much greater import in the organization of the entire venter of the embryo than we have hitherto supposed.

Kemp (1946) has changed various parts of the endoderm in relation to various other parts of the endoderm. He has removed sectors of the floor of the gastrocoele in the gastrular stage and has interchanged these either by cutting off one part of the embryo and then joining to it another embryo with a definite deficiency, or else by reversing, in some cases, the endodermal sectors in the lower portion of the neurula. These have given rise to a set of rather interesting results, for in the course of this interchange he has been able to produce defects of the liver and of

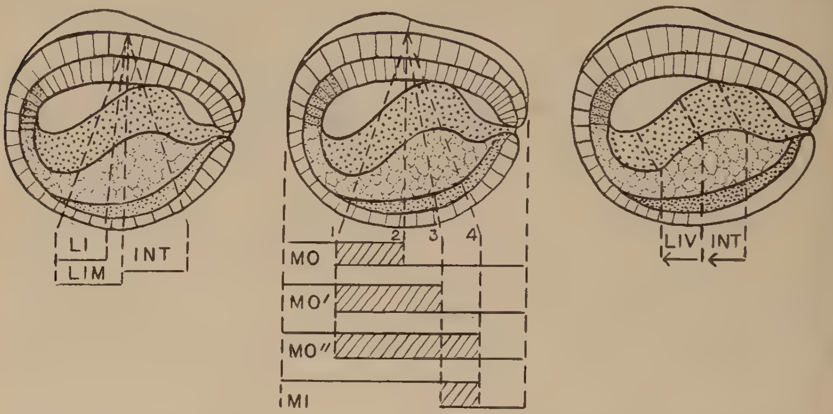


FIGURE 2. These three figures are redrawn from KEMP, 1946. The left figure shows the regions removed, comprising three series: Li, the anterior fourth of the mesenteron; Lim, the anterior third; and Int, middle third. The majority of the Li had functional tracts; Lim, non functional guts, all shortened, esophagus reduced or absent, liver and pancreas reduced, many heart defects; Int, ⅓ regulated completely, 92 per cent had functional tracts.

Middle figure—alteration of yolk endoderm arrangement by transection and reuniting of different levels resulting in duplication of parts of the mesenteron; MO, duplication of anterior third of mesenteron; MO′, anterior half; MO″, anterior two-thirds; MI, post segment. MO—80 per cent functional, 48 per cent completely regulated, enlarged or double pancreas, duplicate gall bladders. (Table 2—KEMP.)

Right figure—shows the regions rotated to reverse A–P axis of the segment. The effects in this series are most marked after the reversal of the Liv segment as indicated by the percentage of abnormality produced. His conclusion is that each endodermal anlage is histologically determined but is equipotential within itself.

TABLE 1*

RESULTS OF DUPLICATION

Series	*Number in series*	*Per cent showing complete regulation*	*Per cent with functional tracts*	*Per cent with duplicated pancreas*	*Per cent with duplicated liver*
MO	25	48	80	20	0
MO′	35	5.5	23	62	6
MO″	32	0	25	100	6
MI	7	13	86	0	0

* From Kemp's Table 2, 1946.

the heart. His results are interesting in showing that a certain regulation can take place at this stage in spite of the way in which the endodermal material has been combined.

As mentioned above, Holtfreter considers that the endodermal cells are polarized at the time they pass through the blastoporal lip and that from then on the endodermal cells in the floor of the archenteron are effective in stretching and accommodating themselves to the region of the floor, passing out and forward as well as laterally, and pulling material from the posterior region into position. They are non-polarized before they enter the blastoporal lip. Their behavior with other cells when isolated shows this polarization complex even though the other cells around them are not reacting, *i.e.*, the cells which normally would encompass the entire mass and make up the dorsal lip and its lateral projections, the marginal zone with its involution; all these can be separated from the action of the endodermal cells in the floor of the archenteron. Holtfreter goes one step further in saying that the surface coat material which is pulled in with the ventral lip as well as with the dorsal lip acts in forming the linings of the cavities of the body as they are later found. This would seem to place a little too much emphasis upon the surface coat, which, although it comes in with the material, is strikingly reduced in the lower layer as compared with its amount on the outside of the egg itself.

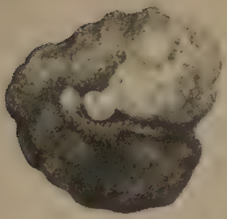
Holtfreter (1933) gave one of the best examples of what can happen in his exo-gastrulation studies. In this case, after having treated the embryos with hypertonic solutions, he secured complete exo-gastrulation with the mesoderm forming its parts lying on top of the endoderm and forming gill arches and body form without the action of the ectoderm. This is an interesting check on the results which Nieuwkoop obtained, for here we have the situation exactly in reverse. The Spemann organizer has not acted in forming the nervous system. Instead, however, during exo-gastrulation, the mesodermal parts have been acted upon, forming a perfectly good head region without, of course, the nervous system, forming gill arches, and forming the body which has the general conformation of what it would have had at the same stage if the embryo had developed with the ectodermal covering around it. The ectoderm, potentially capable of making nervous system as well as body covering, has formed only a bladder of material in which there is no differentiation except that of a common epidermis.

In his 1938 and 1939 studies, Holtfreter has performed other experiments showing still another part of the reaction of the endoderm. When ectoderm and mesoderm are united, a vesicle is formed containing connective tissue, but little else. When the endoderm is added to the isolated bladder of material, there is definite structural conformation and arrangement of the mesoderm, the formation of certain muscular parts in connection with the endoderm, the whole being surrounded by ectoderm. The ectoderm in this situation does not influence the mesoderm nearly so much as does the endoderm, for in the endodermal parts we find a definite uniting of structure, with the mesoderm contributing a muscle layer and the outside sheet of the gut which is differentiating from the endoderm. Here, the potentiality of the gut region is clearly marked; it has a much greater definitive potency for calling forth from mesoderm a differentiation unlike the connective tissue which would develop within the ectodermal bladder by itself. This is another demonstration of the effective facility with which the endoderm acts upon associated structures.

The experiments of Nieuwkoop and Holtfreter strikingly demonstrate that, in the gut endoderm, we have certain capacities for organization which have been neglected in looking upon the embryo as a whole. Its capacity to organize by induction, the facts that the heart is missing without the influence of the yolk endoderm and that the gills are completely missing, had been noted in Stöhr's (1931) experiments. He suspected that the endoderm exerted some influence upon the reacting structures in causing the formation of heart. He did not, however, suppose that the endoderm was directly responsible for smooth musculature of the gut and that the somatopleure and splanchnopleure together could form interchangeable structures.

It is interesting, therefore, to review the morphogenesis of the muscular system, for the splanchnic mesoderm, if not in contact with endoderm, will form striate muscle under the influence of the somatopleure. Any part of the marginal zone which is potent to form muscle striate in character can be induced, according to Bacon's results, to form heart in the presence of endoderm. The reversal of the limb bud (placing the splanchnopleure on the outside, the somatopleure on the inside) gives rise to a perfect limb. All these things tend to show that there is a dominant action exerted by the endoderm which has a part in the induction of muscular structure. It also shows that the final determination of such structure lies not only in its general position, but also in its definitive relationship to other tissue influences.

In the light of Holtfreter's (1939) experiments, it was thought advisable to carry the isolation of the yolk back further into the foregrounds of embryonic history than he had done. Yolk endoderm of the late blastula, when grown in culture (Nicholas, 1945), assumes about the same form relationship as would be found in the normal animal. When isolation experiments were performed upon young blastulae, it was found that not enough of the plasmagel of the external coat material was present in the endoderm to permit its attainment of form. The experiments in which ectoderm is combined with yolk endoderm, as shown in FIGURES 3 to 8, are more successful when different proportional amounts of ectodermal-endodermal material were used. When yolk endoderm material is taken from the early blastula and cultivated without an external cover, the results are rather clearly shown (FIGURE 4). The cells aggregate and so does the nuclear and pigment material; there is a fine granular



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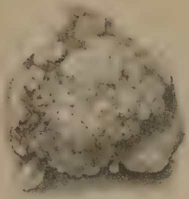

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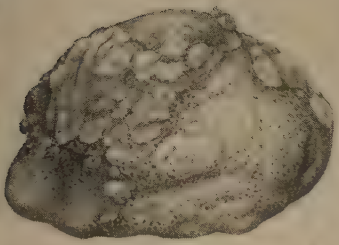

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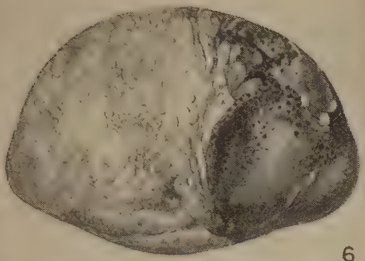

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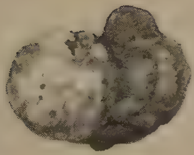

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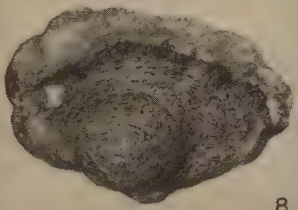

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FIGURE 3. Isolation study of yolk endoderm from early blastula enclosed in envelope of ectoderm. Cultured 5 days. A thin coat covers all of the exposed endodermal material. The ratio of endodermal material to ectodermal cover is 2 to 3.

FIGURE 4. Yolk endoderm of late blastula cultured in the absence of ectoderm in double Holtfreter's solution. Note the cell aggregate and the clumping of pigment and nucleus within the constituent cells. Cultured 4 days. There is no ectoderm present and fine granular material was being exuded from the loose cells located in upper part of the diagram.

FIGURE 5. Yolk endoderm isolate, mid-blastula, in combination with a small ectodermal piece, shown to the left of the figure. The ratio of endoderm to ectoderm is 5 to 1. After 5 days of culture, disintegrative action began, as shown by the loose cells in the upper left of the figure.

FIGURE 6. Yolk endoderm isolated in early blastula and combined with ectoderm in 4-to-1 ratio. There is here, as in the previous figure, a distinct antero-posterior lengthening of the yolk endoderm. There is a rolling of the cells at the inferior margin of the figure, showing that movement of the endodermal constituents continued under these conditions.

FIGURE 7. A 1-to-1 combination of yolk endoderm of an early blastular fragment with ectoderm from the blastular cap. After 7 days of culture, there is no axial or other differentiation, probably due to the small size of the piece.

FIGURE 8. Isolate consisting of 1 part of yolk endoderm combined with 5 parts of ectoderm. The ectoderm contains a small ball of endodermal material which in 6 days of culture did not differentiate.

type of exudate, probably made up of the yolk platelet content, and in spite of the fact that the isolates were kept in double Holtfreter solution, which is osmotically favorable to endoderm preservation, they disintegrate usually within 24 hours. If, however, ectoderm is combined with the yolk endoderm, as shown in FIGURE 3, there is a tendency for the endoderm to receive something from the ectoderm which acts as an external cover preventing the disintegration of both the ectoderm and the endoderm.

The same relationship is shown in FIGURES 5 and 6, which are examples of about the minimum of ectoderm combination which is necessary for the development of the yolk endoderm taken from the early blastula stages. FIGURE 5 shows rather interestingly that the external cover over the yolk endoderm is not adequate to prevent sloughage, which can be seen in the large, loose, white mass of cells to the left of the figure. An isolate (FIGURE 6) in which there was about 25 per cent of ectoderm at the time of its first isolation, has a complete coverage and can be maintained for a considerable period of time. The elongation of the yolk endoderm under these conditions simulates the normal axial relationship. The ectodermal covering is found near the anterior end of one of these masses and near the posterior end of the other, as judged by their general morphological appearance. Ectoderm by itself does not seem to have a definite effect upon the polarization of the yolk mass. An early isolate in about the 128-cell stage is illustrated. Here, an increasing amount of ectodermal material is necessary if the isolate is to survive in double Holtfreter solution. As in previous figures, one sees the relationship of the ectoderm to the yolk endoderm in that there is a consistent smooth covering of material on the outside of the endodermal cells which they themelves do not produce in amounts adequate to cover their surface. In FIGURE 8, also an isolate, in which much more ectoderm was taken and where the relative amount of the endoderm was small, the ectoderm completely covers the endoderm, the endoderm remaining a small, nodular mass inside the ectoderm. While it retains its normal components, there is no indication that there is any development of polarity of axial determination.

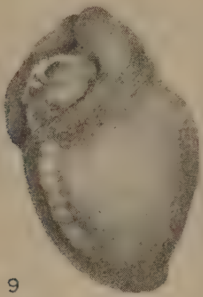
In this series, then, it is shown that material from the early blastula lacks the capacity for the formation of a superficial plasmagel in sufficient quantity to prevent content losses to the surrounding medium (in this case a double Holtfreter solution).

When varying proportions of ectoderm, taken from any region of the blastula, are included with the early blastular endoderm in the isolation, the endoderm can be maintained without disintegration. The self-differentiating powers of the yolk endoderm cannot be demonstrated unless it secures additional material from potential ectoderm cells which increase its surface membrane and enable the endoderm to undergo its own arrangement.

This is not regarded as a direct morphogenetic effect upon the endoderm by the ectoderm, but as a mechanical factor supplied by this material in the form of plasmagel. Elongation and the expression of axial tendencies are obtained only when the yolk endoderm of the early blastula is present in adequate proportions. A four-endoderm-to-one-ectoderm combination, as shown in FIGURE 6, is optimal. When the proportions are reversed, as in FIGURE 8, one endoderm to four ectoderms, the endoderm shows no elongation but remains as a rounded ball with an ectodermal cover.

A separate series of observations was made upon early blastulae in which the roof of the blastocoele was removed, and a piece of the transparent vitelline membrane was cut to fit the opening and inserted so that the ectoderm was fitted around the margins of the cover. It was possible, by this method, to observe the formation of the floor of the blastocoele and the way in which the various parts came into their organization complex during the course of development. The vitelline membrane remains transparent, is retained by the organism without apparent damage to the adjacent tissues, and, since it softens during development, serves to separate the various parts during the critical period in the organization of the floor of the archenteron. It was noted early by this observational method that there was a pattern of cells arising near the midpoint of the blastula in the floor of the blastocoele. There are generally between 16 and 28 of these rather large cells, which appear in the form of the mosaic in the floor of the blastocoele. These are the cells which have formed in the pathway of ingression (see FIGURE 1). Whether they are actually cells that were originally on the outside cannot be definitely stated. There are probably many more cells, not distinctive in either their form or their position, which come in at this time, and certainly the 16 to 24 which projected the pebbled surface on the floor of the blastocoele can be localized as being morphologically in the chain of ingressive material. They later are found under the stomodaeal pit as the blastocoele becomes reduced.

In addition to the experiments reported above, in which the observations of the cells in the floor of the blastocoele could be made, additional experiments were conducted in which the entire cover of the early gastrula was removed. In this series of experiments, the neural folds developed at the marginal limits of the denuded area; the yolk endoderm was exposed, and the results of the previous series of experiments were checked. Just anterior to the midpoint of the blastocoele floor, the pebbled series arises, forming a portion of the floor of the archenteron. Above this develops the stomodaeal pit, while posteriorly the proctodeal pit is found. These two structures seem to develop entirely from the yolk endoderm, which is specific for them. The factors which have to do with the formation of the stomodaeal pit and the proctodeal pit certainly have their origin intrinsically in the yolk endoderm. The occurrence of the lateral neural folds presented somewhat of a problem, since this material was underlain only with marginal zone material which normally would have become lateral mesoderm. When the substrate is marginal zone material, however, and not the usual chorda mesoderm, it has the capacity and competence for forming ectoderm into nervous tissue (see FIGURE 10). The neural folds, while poorly developed anteriorly, show good structural relationships, and the sections through these individuals show perfectly definite systems.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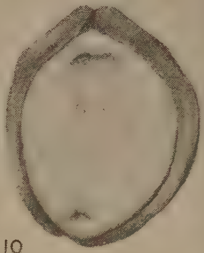
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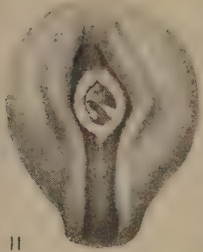
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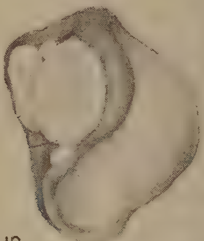
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FIGURE 9. The utilization of the transparent vitelline membrane as a blister through which observations upon the formation of the stomodaeal invagination are made. The neural folds are held apart in the region of the mid- and hindbrain.

FIGURE 10. A culture of the yolk endoderm held in place by the ventral ectoderm and the marginal zone. The operation removed practically the upper half of the blastula. The formation of the proctodaeal and stomodaeal invaginations is regular and occurs because of reactions intrinsic to the ventral half of the early blastula.

FIGURE 11. A small cap was removed from the dorsal part of the blastula and observations made upon the midpoint of the blastocoele floor (*cf.* FIGURE 1). The cellular mosaic developed regularly in the floor and is later, by the involution of the blastocoele, converted into the floor of the archenteron beneath the stomodaeal groove.

FIGURE 12. Culture of early blastula with same type of operation as shown in FIGURE 11 but without vitelline cover. The growth processes in the endoderm are less regular and a part of the endoderm has been displaced in the healing process.

If the experiment is performed later, only part of the roof of the midgastrula is removed and the cellular reactions which are going on in the floor of the gastrocoele can be observed. The nervous system, instead of consisting of half the nervous system in the regions which have been separated by the removal of the roof of the blastula, now is practically complete.

There is a distinct difference, then, in the nervous system effect of the two operations, for in the first case the nervous system forms really as a vestigial type of regenerate; in the second case, where only a small portion of the roof of the blastula was removed, it is practically complete.

Discussion

The question immediately before us to be considered is not that of the localization of the parts of the gut in the yolk endoderm. This is adequately treated in Kemp's (1946) paper. In general, the results given here support Holtfreter's (1939) conclusion that there is early localization of the gut. The stomodaeum and proctodeum are determined in the late blastula, and there are indications that the morphological components which determine this change are the elements which have reached the inside of the blastocoele by ingression. This is a rather important point, for it shows an early localization of gut structure as well as an antero-posterior localization of the materials. The parts, however, between the stomodaeum and the proctodeum seem to have only a general relationship.

The general relationships of the yolk endoderm are more important for the present discussion than the absolute localization of parts such as liver, or the parts of the liver. As has been pointed out previously, the yolk endoderm has a generalized capacity which can call forth from the surrounding tissues certain definite elements. This is also clear from Nieuwkoop's experiments, for, in his study of the origin of the germ cells, one can see the various ancillary actions which the yolk endoderm exerts. It is positive in the formation of the stomodaeum and the heart which shows from Stöhr's experiments (which are the converse of Bacon's), as well as in various experiments having to do with the gills and their formation. The endoderm, then, is exceedingly potent in the formation of structures at the anterior and ventral aspects of the embryo. The fact that splanchnopleure does not develop if the gut is absent shows a definite directive effect of the yolk endoderm upon the histogenesis of what might be regarded as a generalized muscular structure. The inhibiting action of the yolk endoderm which prevents the splanchnopleure from forming limbs is another important indication of its action.

Yolk endoderm is probably the largest source of the chemical materials which will be transformed by the organism. These are the rough products from which the later chemical materials are going to be formed. It is possible that the yolk platelets which have been separated into the ectodermal cells and which are part of their composition are changed in their values with relationship to the chemical constituents by the cells themselves. If this is so, we have a rather clear indication that certain of the materials which are found predominantly in the nervous system later are developing from the raw materials of the yolk endoderm which have been carried about and placed in a definite location by the cellular activity itself. The greater reservoir of these materials still remains in the yolk endoderm which is left behind.

In watching the development of the amphibian forms, one is frequently impressed with the fact that the material found within the gut, consisting of the yolk endoderm particles, which have been enclosed by the gut material, does not completely digest, and, while there is an extraction of material from the yolk, there is still sufficient residue left to make quite a sizable collection of material. This material is relatively inert and should be investigated in order to get the difference between the chemical substances which are involved in the yolk platelet formation and the final residue after its extraction by the organism. This should form a field of rather useful investigation for the chemical understanding of what has gone into the organic relationships which have been formed by the embryo itself.

The yolk endoderm, then, has been shown in the present discussion to be responsible for the formation of the stomodaeum, to take part and be active in the formation of gills, and to possess the capacity for induction of the heart from indifferent mesoderm or from mesoderm which is prospectively significant for the formation of other structures than the heart. Since the yolk endoderm is so active in the formation of anterior structures, it should be looked upon as a possible factor influencing the formation of both the hypophysis and the thyroid. It has a corollary in Nieuwkoop's findings that the endoderm is responsible for the induction of germ cells from the lateral plate. From Nieuwkoop's work, it is clearly shown that the muscle developing from the somites in the splanchnopleuric region will not develop as the smooth muscle of the gut, but acts in the absence of the yolk endoderm as somatopleure which has the capacity for developing limbs at the normal regional level similar to that found in the somatopleure. When the yolk endoderm is removed in early stages, the heart is atypical or absent. When the yolk endoderm is cultured in mid-blastula stages, it elongates, possesses a definite plasmagel coat, and develops the invaginations normal to the formation of a stomodaeum and proctodeum. In earlier stages of the blastula, some material from the outside of the egg must be incorporated along with the yolk endoderm in order to maintain it as a unit and have it continue its development. One can secure the development of practically normal form and extension if the yolk endoderm is 25 per cent of the quantity of the graft and if it is covered by external material. This points to the necessity for the normal operation on the yolk endoderm of material from the outside of the egg. The fact that the mid-blastula stages can be cultured shows that sufficient material of a nature similar to that of the external coat of Holtfreter is present in the cells of the yolk endoderm and can take care of its relationship with the external environment.

The development of the chemical substances and the enzymes which are effective in energy transformation may occur in the cells that carry a maximal or minimal amount of the yolk platelets. These are particularly rich in the endodermal cells, as is shown by the cytochrome oxidase and probably by the adenyl-pyrophosphotase development.

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THE ROLE OF NERVES IN AMPHIBIAN LIMB REGENERATION*

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THE first study of the influence of nerves on regeneration was reported by Tweedy John Todd in 1823.[60] He had discovered that division of the sciatic nerve in the limb of a salamander prevents regeneration distal to the level of division of the nerve. He further stated that division of the nerve causes a regenerate already developing to shrivel and waste. These observations lay unused for many years, until interest revived and Todd's paper was rediscovered early in this century. Several papers appeared soon after the year 1900, some affirming and others expressing doubt[9, 37, 65] that nerves are necessary during regeneration. The question was finally settled in the affirmative by Wolff in 1910[66] and by Walter in 1912.[64] Later, Schotté made it clear that if a nerve supply to a salamander's limb is interrupted, regeneration does not begin until after nerves have regenerated and re-entered the region adjacent to the level of amputation.[44] The expressions of doubt had apparently been due to faulty operations in some cases and to failure to recognize regenerated unmyelinated fibers in others.

Prior to the analysis of the role of nerves in regeneration, attempts were made to isolate the various components, sensory, motor and sympathetic, and to determine thereby which are necessary for regeneration. At first, it seemed that only the sensory supply was necessary because regeneration could occur in the absence of the section of the spinal cord supplying the limb plexus if the dorsal root ganglia and the sensory fibers were not severed.[64] This was regeneration in the absence of a motor supply. Schotté reinvestigated the question of which nerve component, motor or sensory, is needed and found inconsistencies in his results. For example, sometimes the sensory supply alone seemed to be enough for regeneration, at other times a regenerated motor supply was sufficient, and even the original motor supply seemed to be enough in a few cases.[41] It was suspected that the inconsistent failures in regeneration were caused by chance interruption of the sympathetic nerve fibers.[42] Following this hypothesis, attempts were made to eliminate the sympathetic supply in some cases, and in others to remove everything but the sympathetic. The results were never completely consistent, but numbers favored the sympathetic nerves as the important component for limb regeneration.[43]

This conclusion was widely accepted, but it did not go wholly unchallenged. Locatelli pointed out that the sympathetic nerves, especially

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the communicating rami, are extremely small, and the rami, at least, cannot be seen through the dissecting microscope.[20] She refused to accept the conclusion that the sympathetic component is important in regeneration, because of the extreme difficulty of the operations and the failure to confirm them histologically. Her own experiments reaffirmed that limbs can regenerate with sensory fibers alone. Working with the nerves supplying the hind limbs, she dissected around the roots of nerves 17 and 18 in order to remove communicating rami and then pulled 17 and 18 free from the cord. The operation was done in such a way that both ventral and dorsal roots were torn, but the dorsal roots were torn proximal to their ganglia. This left the sensory fibers of the limb still attached to their cell bodies but severed the connections between motor fibers and their cell bodies in the cord. With only the sensory fibers intact, regeneration was normal. In some cases, the sensory component of the 18th nerve by itself supported normal regeneration, but there was no regeneration if the 18th ganglion was extirpated. These results were taken to mean that the 18th dorsal root ganglion had a regeneration-promoting quality peculiar to itself.[20]

This was the state of the problem in 1929. Doubt had been cast on the claims for the sympathetic, and only part of the sensory supply seemed necessary for limb regeneration. In 1942, Singer began to publish an experimental re-analysis of the problem. His first finding was that post-ganglionic sympathetic fibers passing to the anterior limb in *Triturus* do not join the mixed nerves of the brachial plexus by way of communicating rami. Instead, the post-ganglionic fibers leave the sympathetic cord and constitute a separate nerve, the subclavian, closely applied to the subclavian artery.[52] Older observations (see Singer, 1942a, for bibliography) show a subclavian nerve to be the usual pathway for sympathetic fibers leading to an anterior limb in Urodeles. This fact invalidates the earlier experiments on the sympathetics. A reinvestigation showed that complete unilateral sympathectomy in the anterior region did not prevent normal regeneration on the side concerned.[53] In addition, there are reports that forelimbs had regenerated normally after their subclavian arteries had been severed.[14, 44, 67] Since it would be extremely difficult to sever the subclavian artery without also severing the nerve, these observations lend weight to the conclusion that limbs may regenerate without sympathetic nerves.

Further careful re-analysis by Singer, with operations checked histologically, show the truth of the thesis that the sensory is the only component which by itself can support normal regeneration.[53, 54, 55] However, this does not mean that sensory neurones have a peculiar quality enabling them, and them alone, to support regeneration. This is not true, as Singer shows, for, although a normal motor supply will not support regeneration,[55] a regenerated motor supply will.[56] The key to an understanding of this paradox comes from a consideration of the number of fibers involved. There are many more motor fibers after regeneration from a ventral root because the axones branch repeatedly as they regenerate. It has been determined that the number of fibers necessary for limb regeneration in *Triturus* is in the range of one-third to one-half of the total number.[57] The normal motor component does not contain a third of the total number of fibers, but there are enough fibers in a regenerated motor supply to satisfy the threshold requirements. The sensory component is the only normal component with enough fibers to promote regeneration by itself. In fact, there are so many sensory fibers that not all of them are needed. Unless the assumption is made that the regenerated motor fibers have acquired a new quality not present in the normal motor supply, it seems likely that neurones of all components possess the quality necessary for regeneration. Certainly, sensory and regenerated motor nerves do.

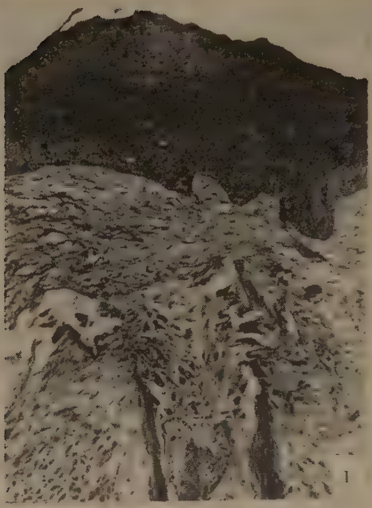
In the course of her experiments, Locatelli discovered that limb nerves deviated from their normal course and, when made to end near the base of a limb, stimulated the development of a new limb over the end of the nerve.[19] The idea that nerves possess specific morphogenetic potency was suggested, but had to be discarded after a demonstration that limb nerves deviated to adjacent territories stimulated regeneration of structures whose form was determined by the territory rather than by the nerves.[11, 2] For example, when the limb nerve ended at the base of the dorsal crest, a piece of dorsal crest developed.

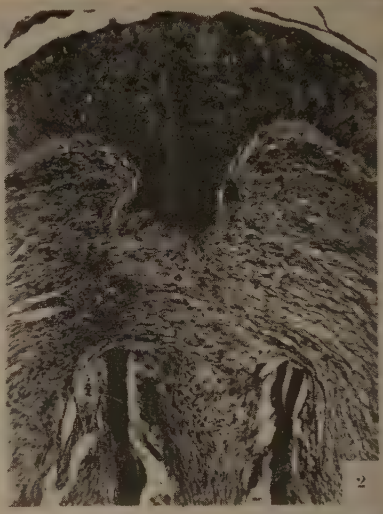
We now face the problem: How do nerves stimulate regeneration? In trying to discover their function, we are aided by the knowledge that embryonic limb buds can grow and differentiate without a nerve supply, as first demonstrated by Harrison.[13, 12] Since nerves are not necessary in embryonic development, it would seem that our search should begin with that phase of regeneration which differs from embryonic development. That phase is the first phase in regeneration, really a preparatory one, during which the old tissue organization at the level of amputation is lost, and free cells, apparently released from old tissues, collect and form a bud.[3, 45, 21, 62, 31, 28, 6, 59] This change from organization as tissues to a cellular type of organization is known as dedifferentiation. The bud or blastema, once it is established, resembles an embryonic limb bud, and from that time on its development is similar to the development of an embryonic limb. It is here, during the preparation of the blastema, that we shall first look for an influence of nerves.

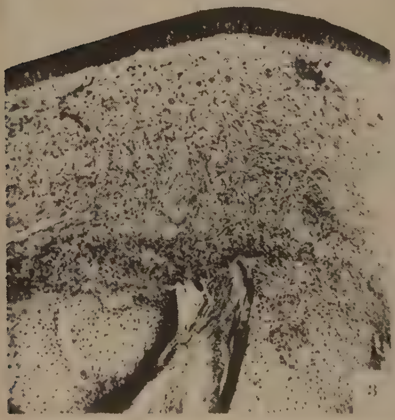
The fact that nerves are required during this early phase of regeneration is clear: a blastema does not form in the absence of nerves. Only lately has there been much work on the causes of failure to regenerate after denervation. Schotté[44] and, later, Butler and Schotté[5] and their coworkers[50, 51] demonstrated that denervated limbs of young, larval salamanders begin to dedifferentiate in the region of an amputation surface but fail to regenerate because dedifferentiation and resorption continue unchecked until whole limbs disappear. The reason for failure to develop a blastema is quite different in older limbs and it is with them that we shall begin our analysis.

It is necessary to digress for a moment in order to introduce a regenerative process which we shall show to be influenced by nerves. It has been known for a long time that there is no regeneration from a wound sealed with skin.[61, 58, 8] This is not the result of a physical block to blastema growth. What a complete skin seal does is this: By covering a wound, it prevents an epidermal epithelium from spreading over and into the wound, thus barring close contact of epidermal wound epithelium with underlying tissues. In normal regeneration, it is the close contact of wound epithelium with underlying tissues which causes histolysis of those internal tissues and the release of free cells as shown by Jeffimoff[15, 16] and by Polejaiev.[23] A complete skin over a wound prevents regeneration, not by preventing growth of a blastema—far from it—but because it prevents dedifferentiation which provides the cells for the blastema. If a limb wound is covered in such a way that one corner of a skin seal is free, a wound epithelium can migrate in under the skin covering the wound and do its work.[15] Intense internal histolysis leading to blastema formation results and, instead of the skin acting as a physical barrier to growth, it, too, undergoes histolysis.[8] Not all epidermal wound coverings, even in salamanders, cause extensive dedifferentiation of the tissues underlying them. For example, although epithelia from limb, tail or abdomen will support limb regeneration, wound epithelia provided by skin of the head or back will not.[16, 29]

Passing, now, to a non-regenerating animal, the adult frog, we find that one of the causes for failure to regenerate is a change in the epidermis during metamorphosis.[24, 49] Miss Gidge and I obtained good regeneration of forearms and wrists in adult frogs after substituting tadpole skin for their own adult skin.[7] A wound epithelium provided by the transplanted tadpole skin migrated over and into the wound of amputation and initiated extensive dedifferentiation. This is very limited in adult frogs if their own epidermis contributes the wound epithelium.[36] The heavy dermis of adult frogs may also be a factor in preventing regeneration because it, along with epidermis, soon closes over a wound, forming a barrier between epidermis and underlying tissues.[33] However, adult anuran limb epithelium can be stimulated by treatments with NaCl[32, 33, 35] or with other irritants[26, 27] and will then act as a younger epithelium and, in its turn, cause dedifferentiation even of dermis with the concurrent release of cells to be used in a blastema. FIGURE 1 is a section of a salt-treated frog limb. If it is compared with FIGURE 2, a control limb of the same age, it will be seen that dedifferentiation had just started in the salted limb. Bone, for example, had begun to erode away. Comparison of a salt-treated limb (FIGURE 3) and a control limb (FIGURE 4) a few days later shows intense dedifferentiation and concurrent blastema formation in the salt-treated limb while the control has already







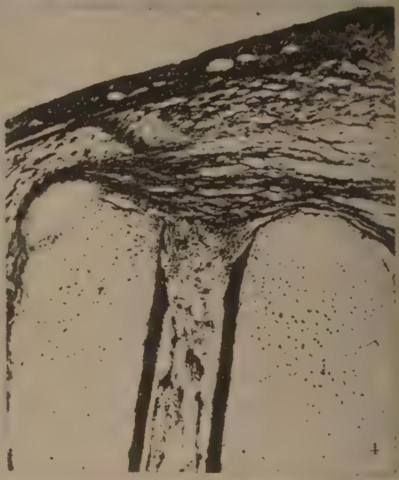


FIGURE 1. A longitudinal section through the tip of a salt-treated frog's limb 19 days after amputation and 10 days after the last salt treatment. Internal dedifferentiation is just beginning. Erosion of bony matrix and the presence of several large, darkly stained, free-fused osteocytes may be noted. X70.

FIGURE 2. A longitudinal section through the tip of an untreated frog's limb 19 days after amputation. This shows no active dedifferentiation and is to be contrasted with the salt-treated limb in FIGURE 1. X70.

FIGURE 3. A longitudinal section through the tip of a salt-treated frog's limb 27 days after amputation and 18 days after the last treatment. A large blastema of mesenchymal cells has formed, beneath which is a zone of dedifferentiating old bone and new cartilage. X45.

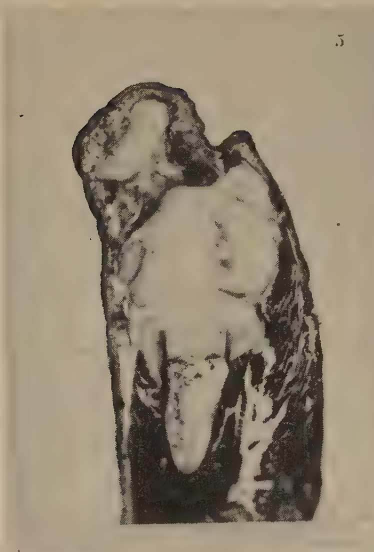
FIGURE 4. A longitudinal section through the tip of an untreated frog's limb 27 days after amputation. Already, fibrous scar tissue and cartilage, flanking the bone, have completely differentiated. This premature differentiation is in marked contrast to the undifferentiated state seen in FIGURE 3. X45.

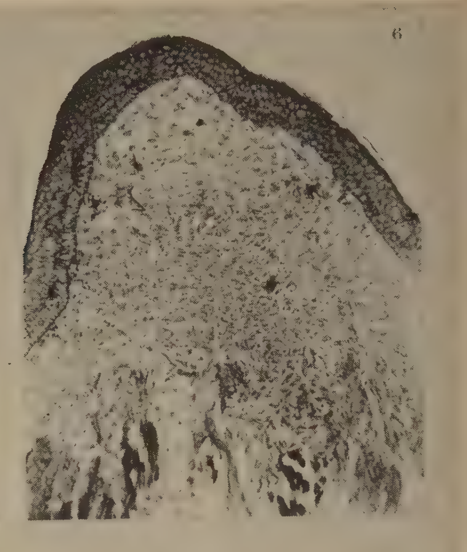
healed with a cartilaginous callus surrounded by fibrous scar tissue. The dedifferentiation had apparently been initiated by a stimulated epithelium.

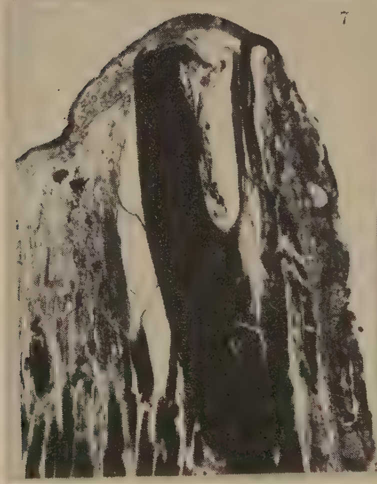
The best of the regenerates obtained from adult frogs by either salt treatments or tadpole skin transplantation, after amputation through the forearm, had normal new forearms and wrists but were abnormal in the more distal regions (FIGURE 5). We shall consider the cause of this abnormality later. At present, we are interested in salt treatments only because of their action in stimulating a wound epithelium.

Returning from the digression with the knowledge that an active wound epithelium causes internal histolysis, we can now show that nerves are normally involved in the activation of the wound epithelium in adult salamanders, as Miss Oliver and I[22] have recently demonstrated. FIGURE 6 shows a blastema on a normal limb of the newt, *Triturus viridescens*. Contrasted with this is the tip of a non-regenerating denervated stump of approximately the same age (FIGURE 7). In the denervated limb, there has been very little dedifferentiation. Only the cut muscle fibers have degenerated. Bone, a good landmark, remains intact to the end of the stump. This stump failed to develop a blastema in the absence of nerves because it failed to dedifferentiate. Notice the wound epithelium, only a few cells thick and composed almost entirely of squamous cells. Now compare a completely denervated limb of the same age which had received baths in a strong NaCl solution (FIGURE 8). The wound epithelium is much thicker and extends down into the wound. This time, there is active dedifferentiation. Bony matrix is being eroded away and, in every case, multi-nucleate, fused osteocytes lie in the wake of the process. The conclusion is drawn that nerves promote dedifferentiation in the adult limb stump.

Dedifferentiation is not the only process for which nerves are needed during regeneration, as we learn from consideration of the ultimate fate of the limbs in question. Cells in the blastemata of normal limbs grow rapidly and remain undifferentiated until a large group of cells has formed. Only then does differentiation begin. In the denervated, salt-treated limbs, there is appreciable dedifferentiation, but the most striking phenomenon is the rapid differentiation of new tissue, cartilage, and fibrous scar tissue, with a concurrent failure of growth. In the untreated, denervated limbs, few cells dedifferentiate, but the few which do so almost immediately redifferentiate again into scar tissue and cartilage. In FIGURE 9, showing a denervated limb of the same age as those in FIGURES 7 and 8, premature differentiation of cartilage along the edge of the partially eroded bone may be seen. This is never observed in salamander limbs with a normal nerve supply. It would seem that nerves promote growth without differentiation, or, rather, shift the balance between growth and differentiation toward growth. In this way, a sufficiently large mass of tissue is available to form a limb by the time differentiation begins.







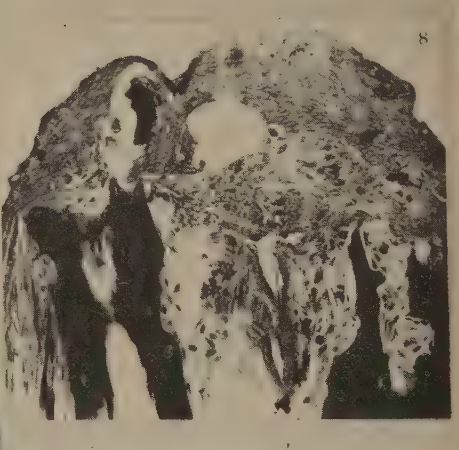


FIGURE 5. One of the best of the frog limb regenerates obtained after transplantation of tadpole skin. The limb had been amputated through the middle of the forearm. Pictured here is the regenerated distal forearm and normal functional wrist. It is to be noted that in this and in all of the induced regenerates the distal part of the hand and the fingers are either abnormal or missing. X12.

FIGURE 6. A cone-stage blastema on the forearm of an adult *Triturus* 23 days after amputation. Dedifferentiation had progressed as far as the tissues at the base of the photograph, and rapid growth of the blastema had begun. X70.

FIGURE 7. The tip of an adult *Triturus* forearm 23 days after complete denervation and amputation. There has been very little loss of structure. Cut muscle fibers have degenerated, but bone is still intact even at the level of amputation. X70.

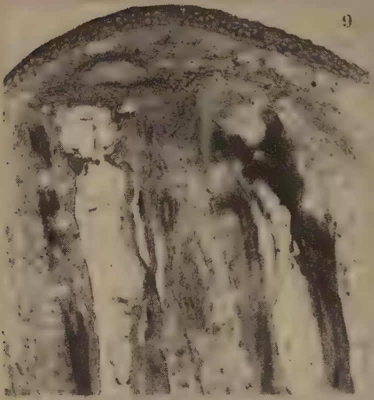
FIGURE 8. The tip of a salt-treated adult *Triturus* forearm 23 days after complete denervation and amputation. The limb had been treated with strong NaCl solutions for the first 14 days after the operation. The greatly thickened wound epithelium, typical for treated limbs, is to be contrasted with the thinner wound epithelium on the untreated limb in FIGURE. 7. Dedifferentiation of bone had begun in the treated limb. X70.

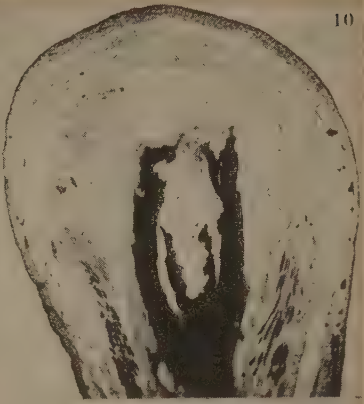
There is supporting evidence for this belief. If a denervated newt limb is re-amputated after 23 days, some nerve fibers have apparently re-entered the limb and a small fraction of these fresh stumps will regenerate limbs, but most of them are insufficiently innervated. These produce callus and scar tissue, as seen in FIGURE 10. The same type of growth was observed by Walter many years ago[64]; it was also caused by insufficent innervation. There can be little doubt that nerves are important for limb regeneration not only because they favor dedifferentiation but also because they promote rapid growth of the dedifferentiated cells. If growth fails and differentiation begins prematurely, all the cells in the vicinity of bone are transformed to chondrocytes and others closer to the periphery become fibroblasts.

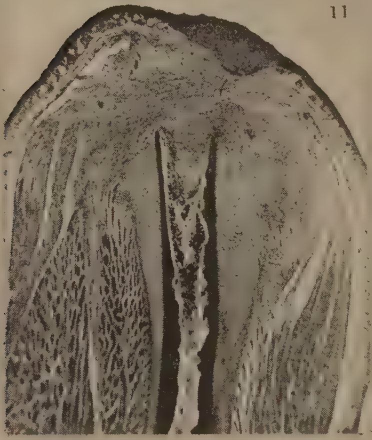
This combination, inadequate dedifferentiation followed by poor growth and rapid dedifferentiation ending with callus formation, is found not only in denervated and x-rayed limbs, but also in the limbs of naturally non-regenerating vertebrates.[34, 35] FIGURE 11 is a section through a 9-day stump of a frog's limb. Already, its limited dedifferentiation and premature differentiation have resulted in the formation of callus and scar. The same picture is seen in a lizard limb stump (FIGURE 12). Here, too, fiber and cartilage form prematurely, sooner than these same tissues form in the regenerating tail of a lizard, as shown by Barber.[1]

The question naturally arises: Do these limbs fail to regenerate because of insufficient innervation? An answer in the affirmative might be closer to the truth if the question were worded: Have the limb tissues of the non-regenerating vertebrates evolved in such a way that the work which nerves do has become more difficult? Changes which occur in anuran tissues at metamorphosis and which cause failure to regenerate are reviewed in an excellent article by Polejaiev in the *Biological Reviews* for 1946.[28] It might be possible to overcome these changes in some of the non-regenerating forms by increasing the quantity of nerve fibers in the limb. There has already been initial success along this line. Singer reported in a discussion, late in 1946, at the A.A.A.S. meetings in Boston that he had stimulated partial limb regeneration in adult frogs by deviating the sciatic nerve to the forelimb. We are awaiting the results of our own attempts to stimulate limb regeneration in lizards by increasing the amount of neural tissue.

Returning again to the salamanders, we find that the need for nerves is not over as soon as a blastema has formed, as demonstrated by Weiss,[67] Schotté,[44, 46, 47] and Samarajew.[38, 39] If nerves are sectioned in a salamander limb during the early stages of blastema formation, the blastema regresses. Even after a regenerate has advanced to the stage when the first form changes are apparent, interference with the nerve supply may prevent the formation of distal structures or cause them to be abnormal.[44, 38, 39] The structures affected are the last to take form. Their formation is ordinarily accompanied by rapid growth. When the nerves







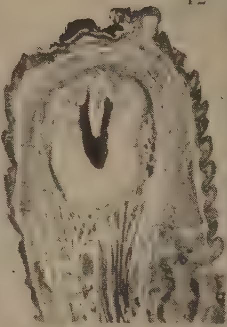


Figure 9. An untreated *Triturus* limb 23 days after denervation and amputation. In this denervated limb, there had been slight erosion of bony matrix, but this was almost immediately followed by differentiation of cartilage, which may be seen at the extreme right of the photograph along the shaft of the bone. There is another small island of differentiating cartilage to the left and near the middle of the bone on the left. X70.

Figure 10. An untreated *Triturus* limb 4 months after a reamputation which had been performed 23 days after the original amputation and denervation. This limb, which probably had some regenerated nerve fibers at the time of reamputation, has formed a cartilaginous callus covered by fibrous scar tissue. X35.

Figure 11. A normal untreated stump of a frog's forearm. Here, as in FIGURE 10, can be seen the cartilaginous callus and the fibrous scar tissue. X22.

Figure 12. A normal untreated stump of a lizard forearm. This naturally non-regenerating limb shows the same arrangement of cartilaginous callus and scar tissue as seen in the non-regenerating frog's limb (FIGURE 11) and in the non-regenerating partially innervated newt limb (FIGURE 10). X12.

are sectioned, the growth rate decreases markedly. It seems likely that these distal abnormalities result from failure of growth at this critical period. Differentiation unaccompanied by rapid growth also seems to be the cause of distal abnormalities in induced anuran regenerates.

The various treatments so far used to artificially stimulate regeneration have provided large amounts of dedifferentiated material but have not increased growth during the period of finger formation when growth is so necessary a part of morphogenesis. Again, we see the adult anuran limb failing in a process which is supported in the urodeles by nerves.

As mentioned earlier, the conditions of failure to regenerate are different in young larval limbs after denervation. Contact between wound epithelium and internal tissues is made, but no fibrous membrane develops under the wound epithelium. Instead, the wound epithelium continues to exert its histolytic action until the entire limb has disappeared. As in adult limbs, there is no growth of the products of dedifferentiation into a regenerate in the absence of nerves. However, a regenerate forms from these dedifferentiated tissues very rapidly when nerves re-enter the region.[47] A partial explanation of the difference in response of young and older animals is that older tissues have acquired a greater propensity for fiber formation.

These differences between young and old denervated limbs are paralleled by those exhibited in x-rayed limbs. Sufficient dosage to prevent regeneration of larval limbs is followed by dedifferentiation and resorption of entire limbs.[3] Not so with older limbs; x-rayed and amputated limbs of large axolotls soon develop a scar beneath the epithelium and fail to regenerate. The argument that a fibrous scar acts as a barrier between epithelium and internal tissues, thereby preventing dedifferentiation, is supported by two observations from the irradiation experiments. X-rayed appendages of axolotls which had been amputated and had already formed a scar beneath the wound epithelium, were made to regenerate normally by removing the scar.[18] The second observation is that young limbs x-rayed twelve days after amputation do develop a fibrous layer beneath the wound epithelium and do not dedifferentiate.[4] In this case, a large blastema was already present and its cells were about to differentiate when the limb was x-rayed.

No theory of nerve action during regeneration can stand unless it can encompass two reports which are, at first, disconcerting, namely, that after transplantation of limbs nerves are not necessary for regeneration. It would seem unwise to doubt these reports, coming as they did, independently, from two of the best laboratories for the study of regeneration. If we accept them as true, and I think we must, our theory must fall unless transplantation itself causes the changes in a limb which we attribute to nerve action. It is fortunate that Polejaiev transplanted tadpole limbs of the same stage as Schotté and Harland denervated. Polejaiev reported regeneration in the transplanted limbs before nerves had an opportunity to re-enter them,[25] while Schotté and Harland, without transplanting, found no regeneration after denervation.[48]

What does transplantation do to a limb? The answer is found in a quotation from Polejaiev and Ginzburg[30] (see also[17]): "In a control at stage IIa subjected to simple limb amputation, the tissue differentiation is preserved in its essential features: the skeleton does not disintegrate, the muscle patterns maintain their integrity; mesodermal cells of the regeneration rudiment do not accumulate on the amputation wound surface under the epithelium. In case of limb autotransplantation, dedifferentiation of tissues takes place: cartilaginous epiphyses are transformed into dense mesenchymatous thickenings; muscle patterns disintegrate, forming a homogeneous mass of mesenchymatous cells which, assuming a round shape and moving towards the epithelium of the amputation wound surface, form the mesodermal part of the regenerating rudiment."

This is strong supporting evidence for the first part of the theory, namely, that nerves act by causing dedifferentiation (except in very young tissues).

Schneider has also reported regeneration without nerves after transplantation.[40] By transplanting a piece of axolotl limb to the region of the back, regeneration of poor feet was obtained. He also stated that regeneration was faster and better when the sciatic nerve was deviated along with the transplant. These facts are in no way incompatible with the second part of the theory, namely, that nerves also support regeneration by supporting growth without premature differentiation.

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Discussion of the Paper

Dr. E. G. Butler (*Princeton University, Princeton, N. J.*):

One of the points I should like to discuss is whether, at the present time, we have final convincing evidence that regression does not occur in amputated nerveless limbs of adult urodeles. It was my understanding from listening to Dr. Rose that he has found no regression in the nerveless limbs in his experiments. On the other hand, I believe that in unpublished experiments Dr. Schotté has found that regression occurs in adult denervated limbs after amputation and prolonged absence of nerves. I should like to ask Dr. Rose how long he maintained the limbs in his experiments in a nerveless condition. It seems to me that, in this problem, time is a very important factor. It is not so much a question whether regression in an amputated limb takes place during a 30- or 60-day period of nervelessness, as whether it will take place during a 4- or 6-month period. Certainly, extreme regression takes place in larval limbs. Can it be that the situation is so different between the larva and the adult? In my opinion, we should have data from experiments on adults carried over a long period of time before we endeavor to answer this question positively.

It is my understanding from Dr. Rose's paper that he regards the epidermis as contributing to the blastema. It is possible that cells from the epidermis pass into the blastema, but do they form a functional component of the blastema? I ask this question not as a criticism of Dr. Rose's work, but rather for information. One of the most involved problems with which I am acquainted is that of endeavoring to recognize the source of cells which contribute to the regeneration blastema. Is there clear evidence that cells from the epidermis not only enter the blastema region, but also actually take part in regenerative activity?

Lastly, I should like to mention the matter of blastema age and its relation to limb regression. My work on regeneration has been primarily with urodele larvae and I am basing my statements chiefly on these.

I feel that the time has come when we can no longer simply refer to a regeneration "blastema." A newly formed blastema is a far different structure, morphologically and physiologically, than an older one. The blastema is a continually changing structure and at different times exhibits different types of activity. So far as regression is concerned, a young blastema is incapable of preventing a nerveless larval limb from undergoing regression; the presence of an older blastema, however, prevents a limb from regressing. I should like to suggest, therefore, that, in studying the physiology of regeneration, in adults as well as in larvae, the age of the blastema always be taken into account. It seems to me that this is an important consideration in the type of blastema which Dr. Rose is studying in his salted limbs.

Dr. S. M. Rose:

We have kept animals whose limbs had been denervated and amputated for as long as five months. Some of these animals began to regenerate limbs after several months' delay, others formed the type of callus shown in FIGURE 10, and still others formed practically no new tissue. There was no measurable regression in any of these animals except in one case, and in that only after the subclavian artery had been inadvertently severed. Judging with the work of Walter and of Singer as a basis, I think that these groups are different because of difference in nerve number. They are alike in failing to regress. Some other factor seems to be more important in causing regression than a nerve number below that necessary for limb regeneration. It would be very interesting to know whether adult limbs which had been maintained absolutely nerveless for a long time would, in time, begin to regress. As I remember, Dr. Schotté did believe that nervelessness for a long time would be followed by regression. Recently, in a discussion, he stated that regression had been obtained in adults after denervation if the amputation was performed through a regenerate. He did not speak of regression of older denervated tissues. I think that the question of the amount of regression after long periods of nervelessness is still unsettled and that we must await a projected paper by Dr. Schotté which will more fully cover the problem of delayed regression.

In our present work, it is clear that nerves play an important role in dedifferentiation. Without them, there is scarcely any dedifferentiation. This is very different from the situation in young larvae. I do believe that there are real differences between larva and adult; both the denervation and x-ray radiation experiments indicate it. My guess is that a difference originally quantitative, which we see reflected in the ability to produce collagen, is the basis for the resulting qualitative difference between young larva and adult. An initial study of some of the general age changes as they affect regeneration has been made by Miss Goodwin.[10] Much more work on this important problem is needed.

I do not know whether epidermal cells enter the blastema and later differentiate into "mesodermal" tissues. There is direct evidence that epidermal cells pass into the blastema. This has been described by Godlewski,[8] by Hellmich,[13a] by Neukomm,[21a] and by Rose.[35] Godlewski, Neukomm and I were almost sure that we could see intergradations between epidermal cells and cells of the blastema. There is also the experiment by Umanski[63] indicating that a blastema may form from untreated skin transplanted to an x-rayed limb. That skin contained both epidermis and dermis, either or both of which can be suspected. We have unpublished studies from which it appears certain that vitally stained cells leave the epidermis and enter the blastema. Later, a few chondrocytes can be found containing the vital dye. Again, uncertainty creeps in and this cannot be considered evidence that epidermal cells become chondrocytes, because many of the epidermal cells are phagocytized and their stained granules transferred to their mesodermal predators. Hellmich, without using vital dyes, came to the conclusion that all of the epidermal cells in a blastema are destroyed. That may be true, but the observations above and other considerations[35] have kept alive the unproven idea that the epidermis may be one of the sources of blastema cells. A better test of the idea is needed than any used so far.

I fully agree with Dr. Butler that consideration of the age of a blastema is important. I should also like to say that knowledge of biochemical changes during this period would be very useful.

QUANTITATIVE STUDIES ON LOCOMOTOR RESPONSES IN *AMBLYSTOMA* LARVAE FOLLOWING SURGICAL ALTERATIONS IN THE NERVOUS SYSTEM

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Introduction

OVER an extended period of years, the author has been interested in the capacity of various portions of the embryonic nervous system of *Amblystoma* to undergo structural and functional readjustments when subjected to new conditions by means of grafting.

One of the earliest experiments in this connection consisted in substituting trunk spinal segments (6, 7, and 8) from a donor embryo for the brachial segments (3, 4, and 5) of a host embryo. The embryos ranged in age from those with completely closed neural folds (stage 21) to those with a prominent tail bud (stage 30). A histological study of the cords in host larvae, approximately 50 days after the operation, showed that the grafted segments in the new position had undergone a volume increase and cellular hyperplasia which approximated the size and cellular content normally characterizing the brachial region of the cord. The forelimbs in 50 per cent of the cases exhibited normal function and were supplied by a typical brachial plexus, whose segmental contribution took origin from the grafted segments. These and other experiments dealing with the interchange of various spinal segments and the effects upon cellular proliferation, showed that the number of cells normally characterizing a given region is not inherently and irrevocably fixed. Some regions, however, are more plastic than others. Some have a greater "potential" for proliferation than others, regardless of their axial position.*

In some recent experiments (Detwiler, 1945) upon the embryonic brain, the presumptive hemispheres were excised in order to study any possible morphogenetic effects of their absence upon the development of the remainder of the brain, particularly the medulla. It had been shown by Burr (1916a) that the cerebral hemispheres of *Amblystoma* are dependent upon the intactness of the nasal sacs for their complete development. He also showed (1916b) that, when a cerebral hemisphere is removed from embryos,* it will regenerate only if the nasal placode is left intact. The stimulus for regeneration was assigned to the ingrowth of the olfactory nerve fibers, a contention which received support by later experiments (1930).

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* For a discussion of the morphogenetic responses of various spinal segments in altered positions see Detwiler, 1936. Hamburger (1946) has studied the proliferation of cells in mechanically isolated portions of the chick spinal cord, and has discussed the results in relation to earlier findings on the amphibian cord following certain experimental procedures.

My own experiments upon the forebrain corroborated Burr's results in that there was no evidence of regeneration of the hemispheres. In these experiments, which were conducted on Harrison's stage 21±,† the excised region included the presumptive nasal placodes and the eye rudiments. Both unilateral and bilateral excisions were made. Since it was doubtful whether embryos lacking the entire forebrain, eyes, and nasal placodes could lead an independent existence beyond the yolk resorption stage, many were fused parabiotically with normal embryos to serve as nurses and at the same time as controls for the operated components. Others were allowed to develop as "free" individuals.

External malformations in the shape of the head became visible fairly early. The growth of the upper jaw was greatly reduced, resulting in a marked protrusion of the lower jaw (FIGURES 1 and 2). In cases of unilateral excision, this condition existed only on the side of operation (FIGURE 3).

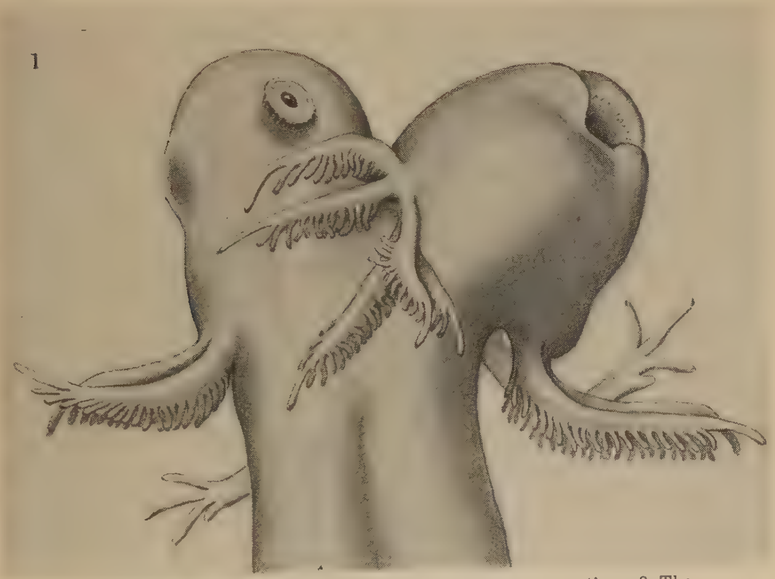

FIGURE 1. Parabiotic twin (FBEBP20) 26 days after operation. x8. The right component lacks both cerebral hemispheres, eyes, and nasal organs.

When the larvae had reached the feeding stage, most of the non-parabiosed individuals, despite the absence of forebrain, eyes, and nasal placode, exhibited snapping reactions when a needle was moved gently

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* Stages of operation not indicated.

† At stage 21 the neural folds are completely closed.

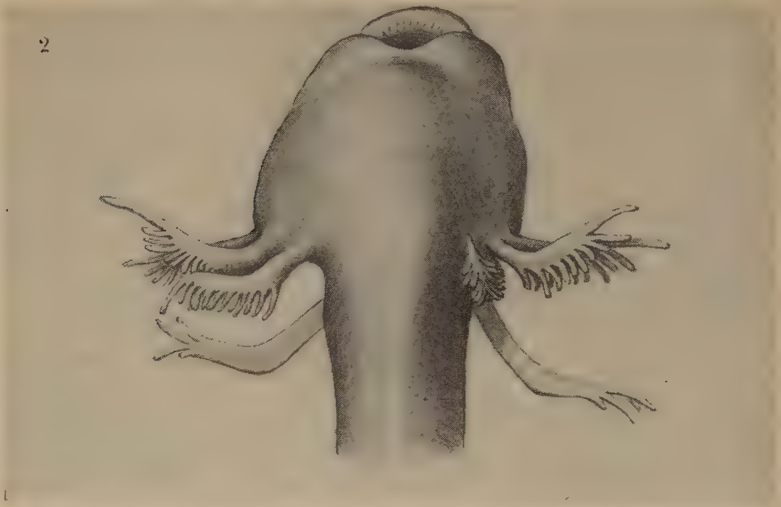


FIGURE 2. *Amblystoma* larva (FBEB44) 26 days after operation. x8. The forebrain, eyes, and nasal placodes were removed from the embryos in stage 21.

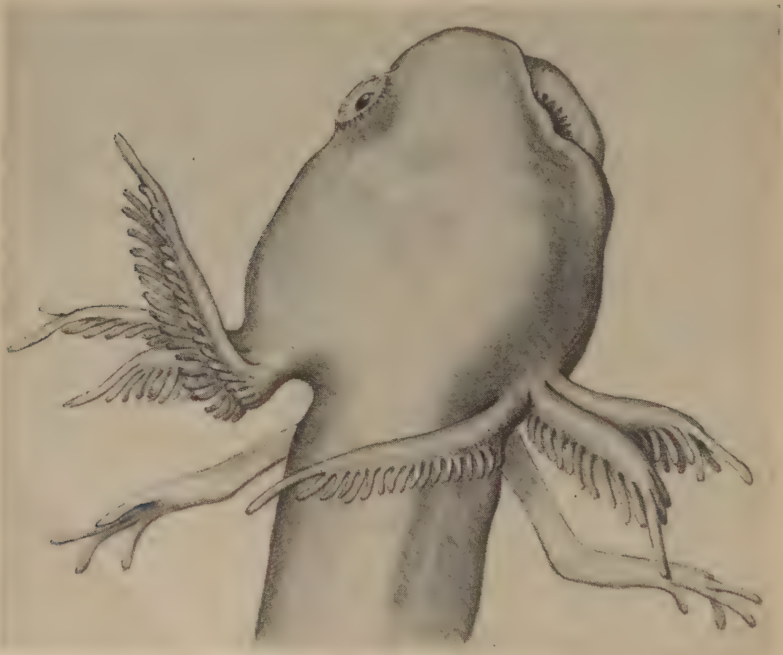
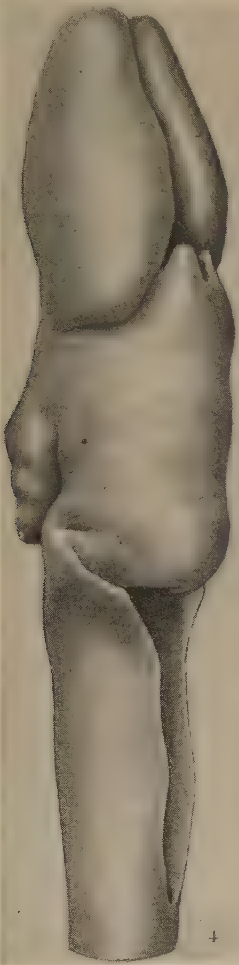


FIGURE 3. *Amblystoma* larva (FBE21) with absence of the right hemisphere, the ipselateral eye, and nasal sac, 30 days after operation. x 8.

back and forth along the side of the head. They were capable also of feeding upon daphnia and small enchytraeid worms. Sharrer (1932) had indicated previously the significance of the lateral line sense organs in the characteristic snapping reactions. It had also been shown later (Detwiler and Copenhaver, 1940) that the growth rate of larvae lacking eyes and nasal organs may keep pace with that of controls under conditions of maximal feeding. This indicated clearly that the lateral line sense organs alone may constitute an adequate receptor apparatus for the detection of food in motion.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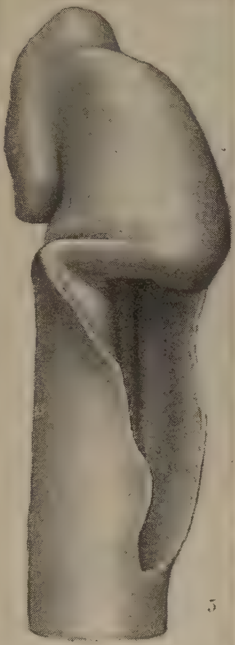
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FIGURE 4. Wax reconstruction of the brain (dorso-lateral view) of the normal component of parabiotic twin FBEB22. x20.

FIGURE 5. Wax reconstruction of the brain (dorso-lateral view) of the operated component of parabiotic twin FBEB22. x20. Note lack of hemispheres and the dorsal portion of the diencephalon.

The intake of food, and the growth of the larvae lacking the forebrain, eyes, and nasal placodes, was markedly curtailed as compared with the controls. Spontaneous behavior in general was greatly reduced, both quantitatively and qualitatively, especially the foraging reactions, regardless of the amount of food available. The mean length of the larvae at 45 days of age was slightly more than 50 per cent of the length of maximally fed normal animals of similar age. Nevertheless, the motor activities concerned with lurching, engulfing food, chewing, and swallowing were carried out in an integrated manner, although they were decidedly less vigorous than in larvae with intact hemispheres. In the parabiotic twins, the eating function was taken over largely by the more active conjoined normal component, but here, too, the operated component was seen to snap and engulf food. Although actual measurements were not recorded for all cases, it may be said that the growth of the twins was greater than that of the operated "free" individuals, but not as great as in the normal control larvae.

Wax reconstructions of the brains of the 2 components in twin 22 are shown in FIGURES 4 and 5. The operated component not only lacks the hemispheres, but the dorsal portion of the diencephalon is also wanting. The hypothalamic region and the pituitary gland (both glandular and neural portions) are present and essentially normal. This situation was characteristic of all cases whether the individual was parabiosed or not. The defective diencephalon is interpreted as resulting from partial ablation during the operation rather than to any morphogenetic influence due to the absence of the hemispheres.

Despite the complete absence of the hemispheres, an incomplete diencephalon, and a possibly smaller mesencephalon, the medulla in all cases showed no significant reduction in size. The medullas of the operated components were somewhat shorter than those of the normal components of the twins (FIGURE 6, *cf.* *A* and *B*), but their volume in all cases studied, except one, ranged from 92 to 99 per cent of normal (Detwiler, 1945, Table 1).

In cases with unilateral excision of the forebrain, there occurred a compensatory enlargement of the contralateral nasal sac and the adjacent hemisphere. The single hemispheres in 4 cases studied showed volume increase ranging from 73 to 88 per cent of the volume of both hemispheres in a control larva of similar length (Detwiler, *op. cit.*, Table 2). A histological study of nuclear size forced the conclusion that the compensatory enlargement of the hemisphere was accomplished by a cellular hyperplasia.

The capacity of the young larvae lacking hemispheres, eyes, and nasal organ to obtain food and to lead an autonomous existence, led to experiments designed to test the behavior of larvae lacking the midbrain.

Nicholas (1930) excised the embryonic mesencephalon and replaced it with a graft consisting of the forelimb and adjacent pronephric rudiments, thus isolating the forebrain from the remainder of the nervous

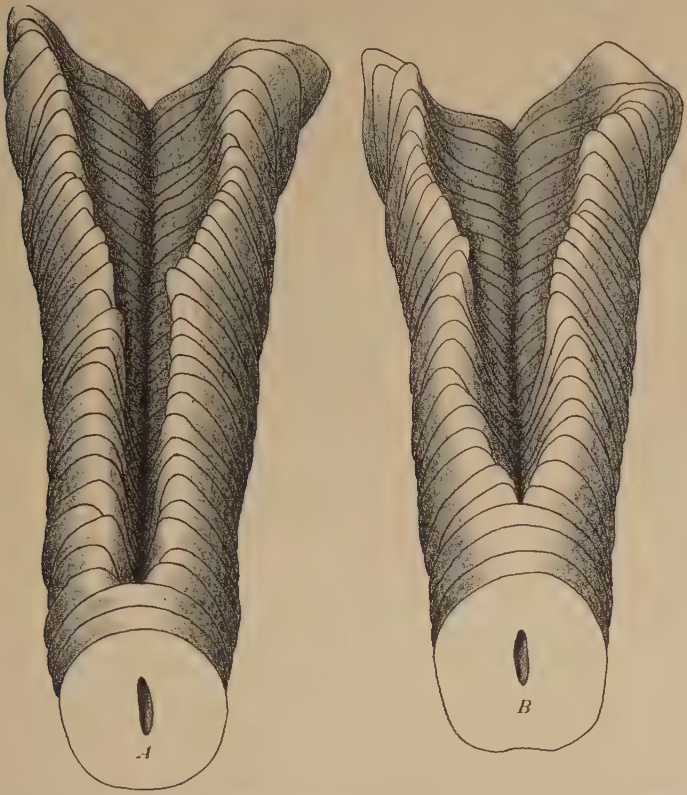

FIGURE 6. Graphic reconstruction of the medullas of normal component (*A*) and operated component (*B*) of parabiotic twin FBEB3. The operated component lacks the cerebral hemispheres, eyes and nasal sacs. x40. (See FIGURE 1.)

system. He found that the early responses (*C* and *S* reactions) were carried out normally. He said little about the effects of the operation upon behavior of older larvae, except that spontaneous movements were not present and that vigorous swimming movements could be elicited by stimulation. These apparently diminished in amount as the animals became older. Nicholas did observe that his larvae were unable to obtain food, and this failure was regarded by him as due to the fact that "the removal of the mesencephalon created a block in the nervous system so that normal feeding responses could not be obtained."*

Experimental Results

In my own experiments upon the mesencephalon, three types were performed: (A) excision of the mesencephalon from embryos in stage 20–21 and replacement by the first and second spinal cord segments from a donor embryo in stage 22–23; (B) end-to-end reversal of the entire midbrain in embryos of stage 22; and (C) unilateral reversal of the midbrain in embryos in stage 20–21, involving reversal of the anteroposterior axis only.

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* In cases where the larvae were kept beyond the feeding stage (stage 46), the animals were fed by artificial means.

Excision of the Mesencephalon and Its Replacement by Cord Segments I and II. This procedure differs from that of Nicholas (1930) in that nervous continuity is maintained between the fore- and hindbrain. The majority of the larvae at the feeding stage were unable to eat. Several exhibited feeble snapping reactions but were unable to obtain their prey. Only 2 cases in 24 were recorded as having eaten, and their intake was very meager. In most cases, the jaws were immobile and held slightly apart. This condition readily explains the inability to capture *Daphnia* in those cases where snapping was attempted. The incidence of kyphosis was very low; there were only 2 cases in this series which developed this condition. This is in marked contrast with the results obtained by Nicholas, all of whose larvae developed a marked ventral bending of the head and tail. The fact that the larvae in the present experiments developed as straight individuals naturally favored a critical study of their swimming responses.

Observations upon Swimming Responses. All the early larvae exhibited normal *C* and *S* flexures and swimming responses. In later stages, however, despite previous manifestations of an adequate intraspinal swimming mechanism, the larvae showed progressive failure both in their responses to tactile stimulation and in their locomotor ability. The method of studying behavior was as follows: Individual larvae (normal and mesencephalonectomized of similar stages) were placed in a Syracuse dish and stimulated 25 times at approximately 5-second intervals. This was done by stroking the skin over the myotomes with a human hair, according to the method of Coghill (1909). At the end of 25 stimulations, the number of "misses" was recorded. In addition to studying the responses to tactile stimulation, the distance traveled in response to 25 stimulations was measured. This was done as follows: A circle, the diameter of which equaled the inside diameter of a Syracuse dish, was drawn upon a card. This was divided into 10 sectors of arc (36° each). The sectors were numbered successively from 1 to 10 both clockwise and counterclockwise, and the card was then placed beneath the dish so that the circle coincided with the inner wall of the dish. A larva was placed adjacent to the inner wall of the dish and headed in a clockwise direction. It was then stimulated and the number of sectors traveled before coming to rest recorded. This was done for 25 stimulations at approximately 5-second intervals. At the end of these, the number of positive reactions and the total distance traveled in units (sectors of arc of 36° each) were recorded for each larva. Young larvae are strongly thigmotactic and nearly always swim along the wall of the dish. In order to avoid occasional short-cutting, the device was improved by placing a glass ring within the Syracuse dish which provided a "moat" about 7 mm. in diameter. The improved device is shown in FIGURE 7.

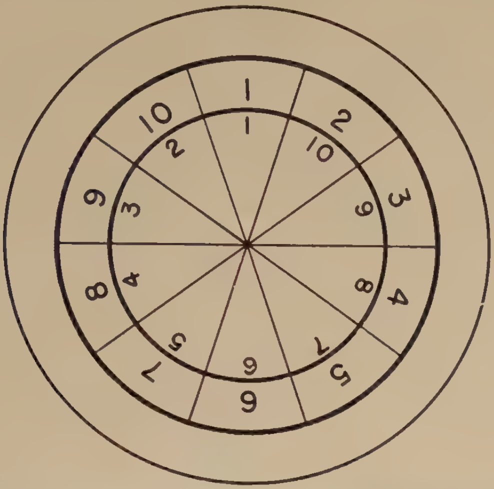


FIGURE 7. An improved device placed beneath a Syracuse dish for quantitating the distance traveled by young *Amblystoma* larvae (stages 39 to 46 and beyond). The outer heavy circle corresponds to the inner wall of the dish; the inner heavy circle indicates a glass ring the height of the dish. The space between the two represents a "moat" approximately 7 mm. in diameter. Each larva was placed in the moat and stimulated 25 successive times at approximately 5-second intervals, and the total distance traveled was recorded in units (sectors of arc). The glass ring has been added to the original device (Detwiler, 1945, Figure 1) to prevent larvae from occasionally short-cutting as they swim along the wall of the dish (*v.* text).

Records were made upon 2 separate groups each consisting of 4 control and 6 experimental larvae. The data on these 2 groups are shown in FIGURES 8, 9, 10 and 11. FIGURES 8 and 10 show the responses to tactile stimulation, while FIGURES 9 and 11 record the average distance traveled by the larvae at the stages indicated. It is seen that, beyond stage 41, the number of "misses" among the experimental animals was indeed very striking. Although there was some variation in the average distance traveled by the normals at the various stages (solid columns), it is clear that the propulsive power of the experimental larvae was greatly reduced (stippled columns). In no single experimental larva from stage 41 on did the maximum swimming response equal the minimum response of any of the normal larvae.

It is apparent from the findings that the execution of normal locomotor responses quite early in larval life depends upon the intactness of the mesencephalon. Up to about stage 40, the absence of this structure appears to have little or no effect upon the swimming activities, which are autonomous in the sense that they apparently can be carried out entirely independent of any structural connection with the midbrain. The rapid falling-off in the locomotor capacity at this period (stage 40–41) is regarded as evidence of the importance of the midbrain tectum

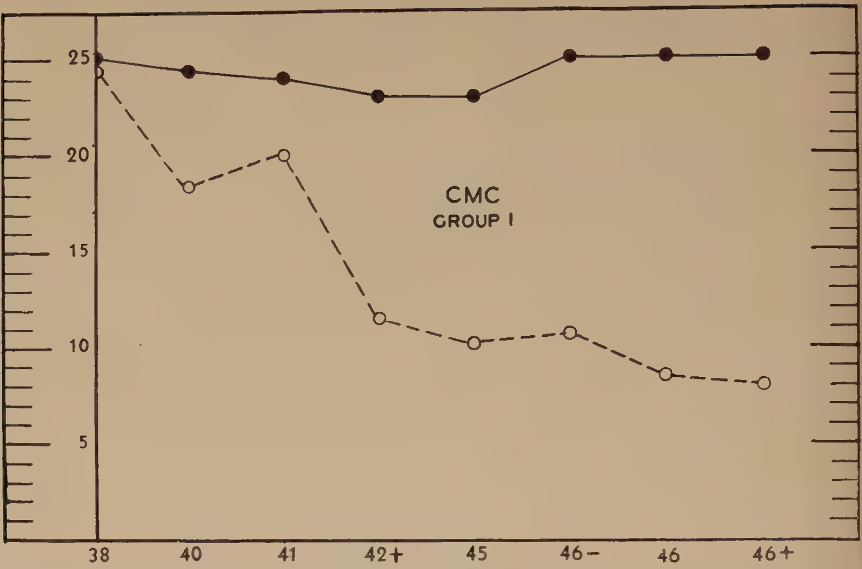


FIGURE 8. Graph showing locomotor responses of *Amblystoma* larvae (CMC-group 1) to 25 successive tactile stimulations at 5-second intervals. Continuous line shows the average for 4 normal control larvae; broken line shows average for 6 larvae in which the midbrain was replaced by the first two segments of the spinal cord. *X* axis represents stage; *Y* axis indicates number of responses.

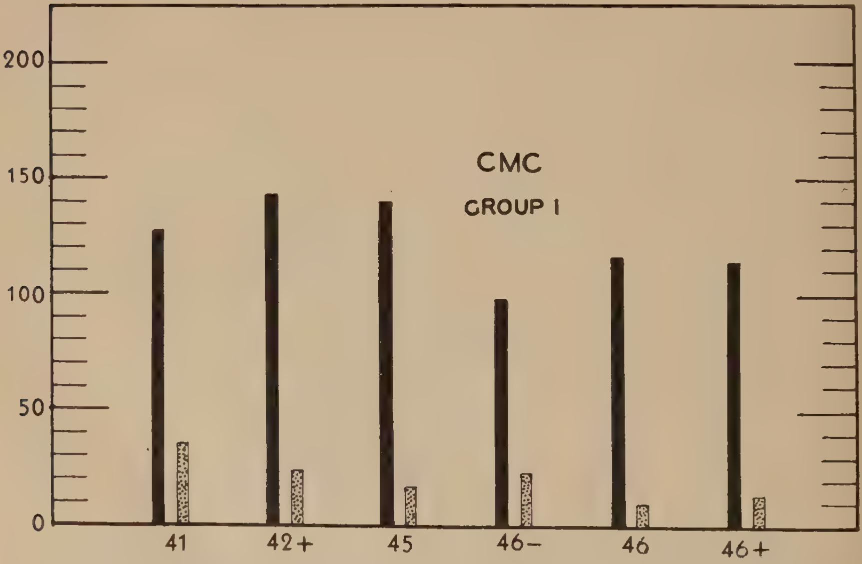


FIGURE 9. Graph showing total distance traveled by the larvae in group 1, in response to 25 successive stimulations at 5-second intervals. The solid columns show the average distance covered by 4 control larvae; the stippled columns show the average distance traveled by 6 larvae whose midbrain was replaced by the first and second spinal cord segments. *X* axis designates stages; *Y* axis represents distance in units (sectors of arc as shown in FIGURE 7).

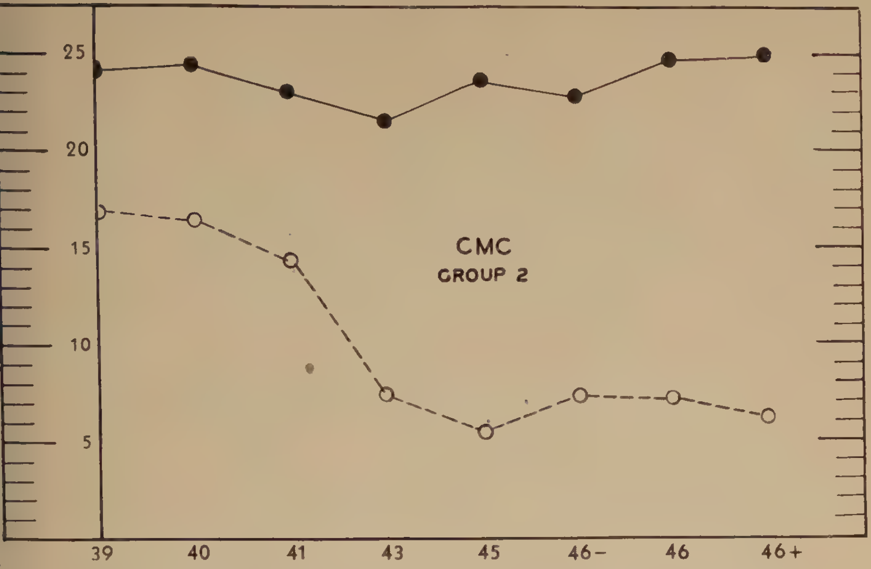


FIGURE 10. Graph showing locomotor responses of the larvae in group 2 (CMC) to 25 successive tactile stimulations at 5-second intervals. Continuous line shows average for 4 control larvae; broken line shows average for 6 larvae with midbrain replaced by spinal cord segments 1 and 2. *X* axis indicates stages; *Y* axis indicates number of responses.

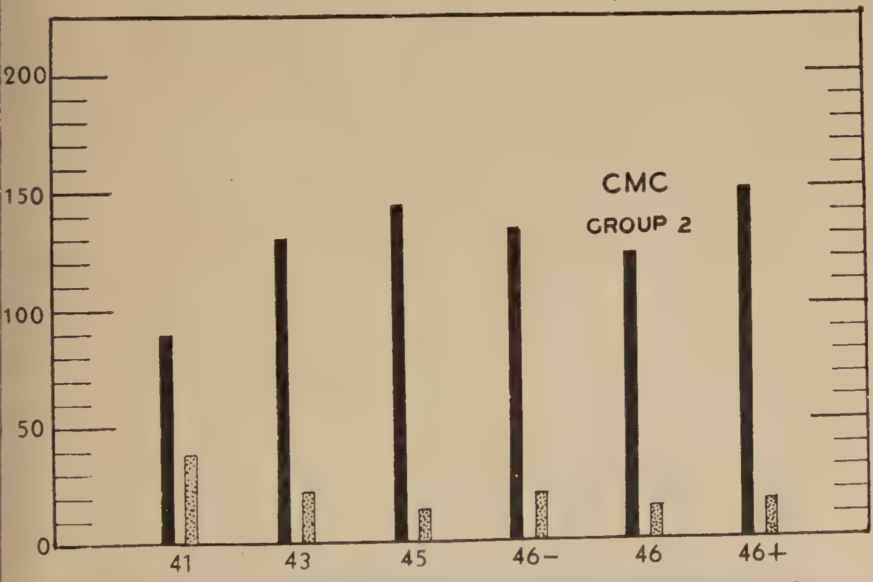


FIGURE 11. Graph showing total distance traveled by the larvae in group 2 (see explanation of FIGURE 9). *X* axis designates stages; *Y* axis represents distance in units (sectors of arc as shown in FIGURE 7).

in coordinating and sustaining these functions. Crossed and uncrossed tracts arising in the tectum invade the medulla as the tecto-bulbar tracts. These make synaptic connections with tracts which arise in the medulla and invade the cord as the bulbo-spinal tracts (Herrick, 1914). However, Herrick (1939) says that some of the fibers arising in the tectum probably invade the spinal cord directly as the tecto-spinal fibers. According to him, the uncrossed tecto-bulbar tracts develop earlier than the crossed fibers of this system. These latter appear in preparations "subsequent to early swimming stages." In the earlier feeding stages, he finds that essentially adult relations have been attained.

It should be pointed out here that larvae lacking the midbrain are still capable of some locomotor activity, *i.e.*, the intra-spinal mechanism can still function, but to a greatly diminished degree (FIGURES 9 and 11). The evidence seems clear, however, that the spinal apparatus, which is entirely adequate for normal propulsion in the early stages (37–40±), later falls under mesencephalic control.

Although no attempt was made to trace out the pre-otic cranial nerves, it would appear that the failure of the animals in this series to eat (*vide supra*) was largely due to the defective development and displacement of the V–VII ganglionic complex as a result of the operation.

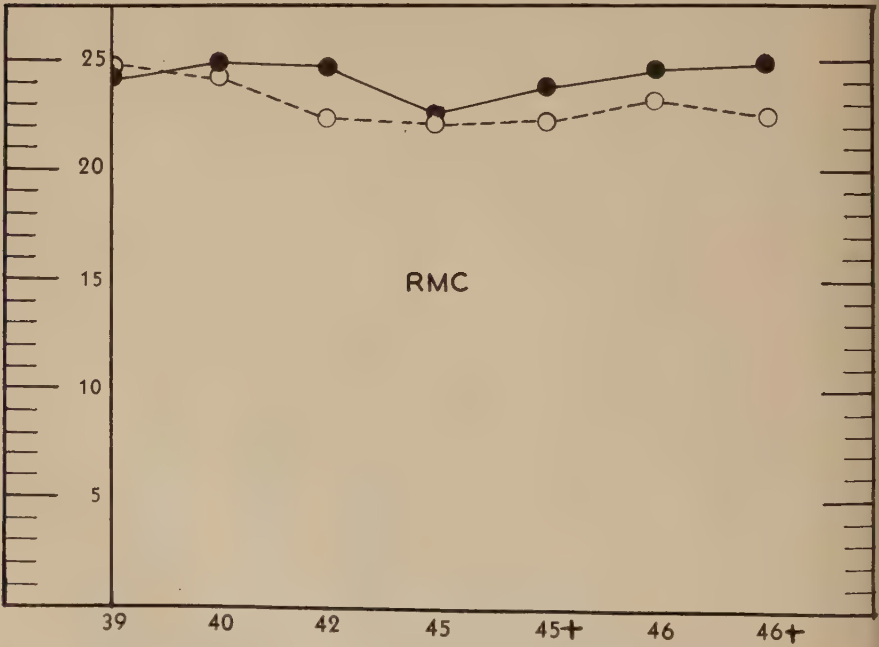


FIGURE 12. Graph showing locomotor responses to 25 successive tactile stimulations at 5-second intervals. Continuous line shows average for 4 normal control larvae; broken line shows average for 6 larvae in which the midbrain was reversed end for end at stage 22. *X* axis designates stages; *Y* axis indicates number of responses.

Failure to cut precisely between embryonic mesencephalon and medulla resulted in the inclusion of the anterior end of the medulla in many excisions—thus involving either the inclusion of or the disturbances to the adjacent V–VII complex.

End-to-End Reversal of the Mesencephalon. As in the series described above, the early *C* and *S* responses were normal. The responses of larvae to tactile stimulation and their motor ability were tested in the manner described above. The results are shown in FIGURES 12 and 13, and they reveal the fact that, despite complete reversal of the midbrain, the larvae responded to tactile stimulation nearly as well as did the controls. More striking, perhaps, is the fact that the motor capacity was only slightly lowered. This is in marked contrast with the greatly curtailed motor capacity of those lacking the midbrain (*cf.* FIGURES 9 and 11). A microscopic examination of the larvae in this series showed that, in 4 of the 9 cases studied, there was no detectable evidence that the midbrain had been reversed (FIGURES 14 and 15). In 5 others, which were incapable of feeding, there were morphological deficiencies which could well account for this defect (Detwiler, 1946a, p. 129).

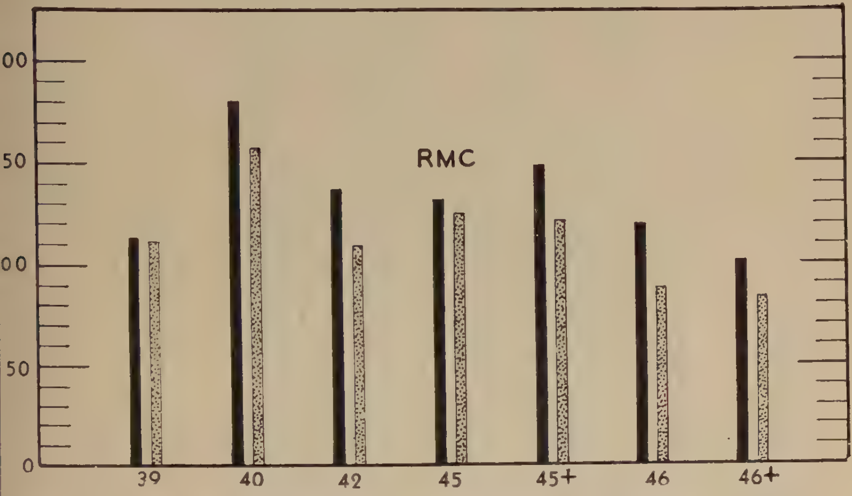
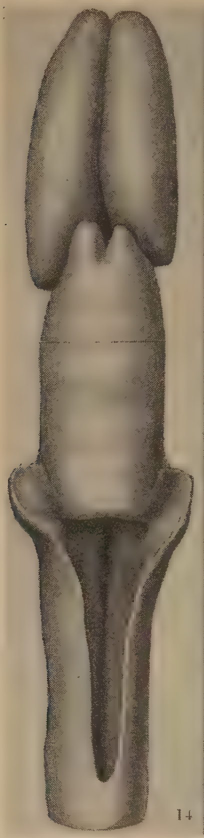


FIGURE 13. Graph showing total distance traveled by larvae in response to 25 successive stimulations at 5-second intervals. The solid columns show the average scores for 4 control larvae; the stippled columns show the average scores of 6 larvae in which the midbrain was reversed end for end in stage 22. *X* axis designates stages; *Y* axis indicates distance in units (sectors of arc as shown in FIGURE 7).

Unilateral Reversal of the Midbrain. In these experiments, the right half of the mesencephalon was excised and replaced by the left half from a donor embryo, thus reversing only the antero-posterior axis. The young larvae, like those of the two previous series, showed normal developmental behavior. No quantitative records were made of the motor activities until stage 46, when 8 larvae were tested along with 6 normal



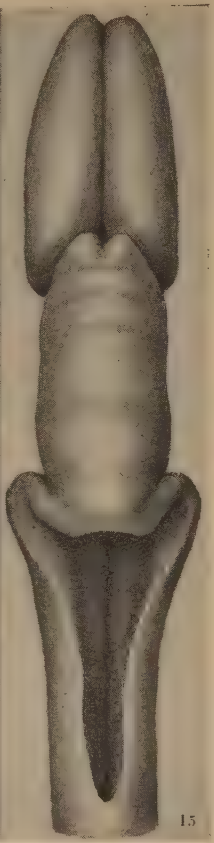


Figure 14. Reconstruction model of the brain of a normal larva (48 days, 35 mm.) for comparison with the brain of case RMC60 (with reversed mesencephalon) as shown in FIGURE 15. x40.

Figure 15. Reconstruction model of the brain of case RMC60 (42 days, 38 mm.). The embryonic midbrain was reversed end for end in stage 22 (*cf.* FIGURE 14). x40.

controls of the same developmental stage. The results showed that the average distance covered by the experimental animals equaled that of the controls. Certain developmental abnormalities were encountered in some of the cases in this series; these have been described previously (Detwiler, 1945, p. 132). One of the most interesting conditions consisted in the failure of the graft to fuse with the cephalic and caudal stump of the host brain. In consequence, the graft developed into an isolated, bilaterally symmetrical whole, and the excised midbrain half was restored by regeneration from the contralateral intact half (Detwiler, 1946b). These cases are of especial interest in showing that, at this stage of development, half a mesencephalon is capable of restoring the whole. The same capacity had been shown previously for the medulla (Detwiler, 1944).

Locomotor Responses of Larvae Lacking the Cerebral Hemispheres. Although the general behavior of larvae lacking the hemispheres had been observed prior to those without the midbrain, no quantitative studies on locomotor responses were made at the time. Consequently, the experiments were repeated and the motor ability tested with the device shown in FIGURE 7. Two groups each consisting of 5 operated and 5 con-

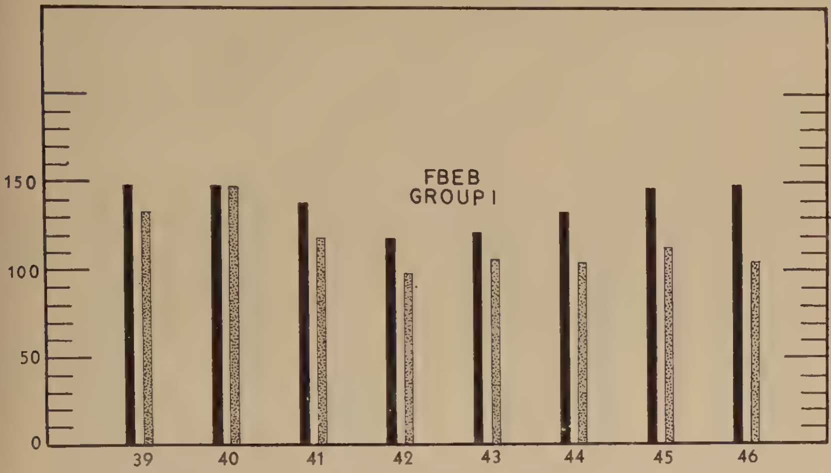


FIGURE 16. Graph showing total distance traveled by the larvae in group 1, in response to 25 successive stimulations at 5-second intervals. The solid columns show the average scores for 5 control larvae; the stippled columns give the average scores for 5 larvae lacking the cerebral hemispheres and the dorsal portion of the diencephalon. X axis designates stages; Y axis represents distance in units (sectors of arc as shown in FIGURE 7).

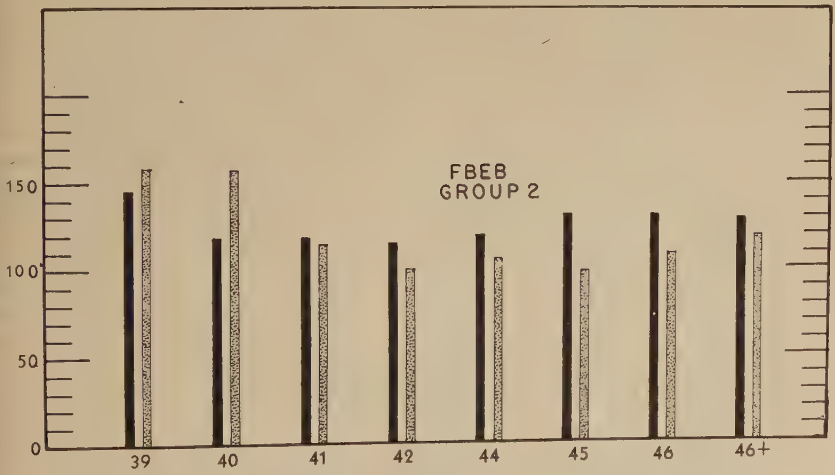
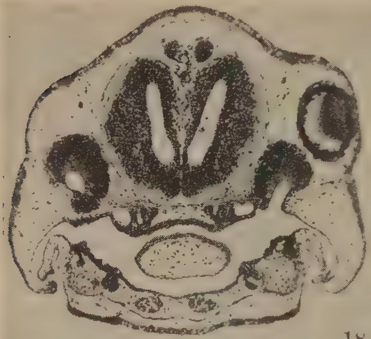


FIGURE 17. Graph showing average scores of 5 control larvae (solid columns) and 5 experimental larvae (stippled columns) in group 2. See explanation of FIGURE 16.

trols were tested from stages 39 to 46+ (feeding stage). The averages of the scores for both normal and operated larvae in the two separate groups are illustrated graphically in FIGURES 16 and 17. These show that the operated larvae in the earlier stages do somewhat better, when compared with their controls, than they do in later stages (stages 45 and 46). The lowered motor ability, however, is only slight as compared with the extreme motor incapacity of larvae lacking the midbrain. The lack of the forebrain also had no effect upon the responses to tactile stimulation. The number of "misses" to trunk tactile stimulation among the operated larvae was no greater than among the controls. This is in marked contrast with the failure exhibited by the mesencephalonecto-



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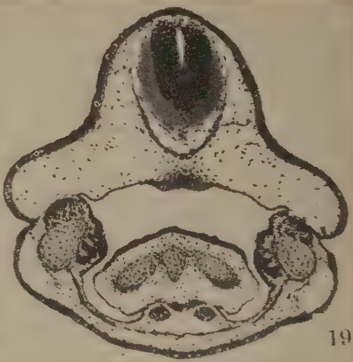

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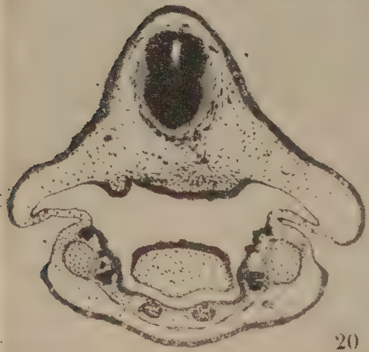

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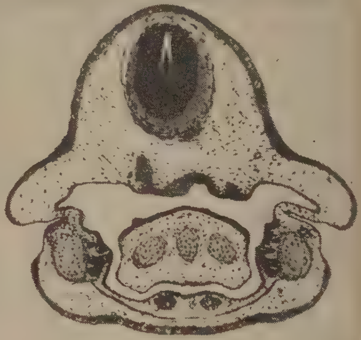

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FIGURE 18. Photomicrograph of transverse section (2-1-4) of a normal larva (CA) at stage 46 (feeding stage) showing cerebral hemispheres, nasal placodes, and an eye. It is the thirty-first section caudal to the first appearance of the brain in section. x33.3.

FIGURE 19. Photomicrograph of section (1-4-1) from case FBEB (4A) at stage 46. It is the seventh section caudal to the first appearance of the brain in section. The larva is completely devoid of cerebral hemispheres and the dorsal portion of the diencephalon. x33.3.

FIGURE 20. Photomicrograph of section (1-3-4) from case FBEB (25A). It is the seventh section caudal to the first appearance of the brain in section. The larva lacked the hemispheres and the dorsal portion of the diencephalon. x33.3.

FIGURE 21. Photomicrograph of section (1-4-2) from case FBEB (25). Conditions same as those described for FIGURES 19 and 20. x33.

mized larvae, which showed a striking decline in response to tactile stimulation subsequent to stages 40 and 41.

Morphological Observations. Ten operated larvae and 1 control subsequent to stage 46 were fixed and studied in serial transverse sections. All 10 cases showed a total lack of the hemispheres. In addition, the dorsal portion of the diencephalon was also wanting. The hypothalamic region and pituitary gland were present in most cases, but in several this region was defective.

Four of the sectioned animals are illustrated in FIGURES 18–21. FIGURE 18 is a photomicrograph of the thirty-first section caudal to the beginning of the hemispheres in a control larva (*A*). The hemispheres are prominent, as are also the nasal placodes and an eye. The microphotographs shown in FIGURES 19, 20, and 21 represent the seventh section caudal to the first appearance of brain tissue in the operated animals. The brain in these sections is readily recognizable as mesencephalon.

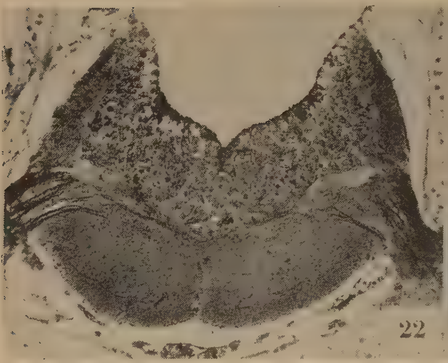
Many of the animals of this series failed to feed. Eight larvae which did eat followed a behavior pattern similar to those described previously (Detwiler, 1945), *viz.*, curtailed spontaneous behavior, rather feeble lurching, reduced food intake, and marked reduction in growth. The activities involved in lurching, engulfing, chewing, and swimming were performed in an integrated manner.

Motor Responses Following the Excision of the Right Mauthner's Neurone and of the Right Ear Vesicle. In teleost fishes and in larval Amphibia, there exist 2 giant neurones known as Mauthner's fibers. These have been of considerable interest to neurohistologists because of certain anatomical features they exhibit—mainly the gigantic size of the cell, the high degree of differentiation, the characteristic position in the medulla, the extensive dendritic connections, the internal cell structure, and the nature of the synapses.

Studies by different authors upon the anatomical connection of these cells in various forms have resulted in several suggestions regarding their function.

In *Amblystoma*, the two perikarya occupy a lateral position in the medulla at the level of the entrance of the VIII nerve (FIGURE 22), and the axones, after decussating in the medulla, course caudally through the spinal cord in a ventral position (FIGURE 23) where connections are made with both motor and intercalary cells (Coghill, 1934).

With regard to the function of this cell, Bartelmez (1915) says: "The reduction of the latent period by elimination of the synapses and the highly medullated character of the system have led me to believe that we are dealing here with a reflex in which speed and precision are very important, and I would suggest that it is this reflex which enables the animal to keep perfect control of its equilibrium in the most rapid and intricate movements." He also suggests that the great caliber of the axones, the broad contact surfaces in the synapse of the lateral dendrite,



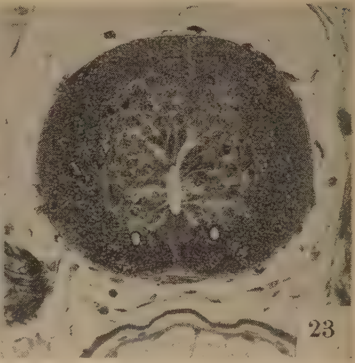


FIGURE 22. Photomicrograph of section (1–3–5) through the medulla of case MPEB (2A) (39 days), showing Mauthner's cells, and their positional relation to the VIII nerve. x50.

FIGURE 23. Photomicrograph of section through the spinal cord at the level of the third spinal nerve showing position of Mauthner's fibers. x66.

and the highly insulated character of the whole path, all point to a very rapid reflex. The fact that Mauthner's cell, in addition to its connections with the VIII nerve roots, has connections with every center in the brain which receives impulses that may be used in equilibrium, strongly suggested to Bartelmez an important role in equilibratory reflexes.

Although much attention had been paid to the dendrite connections of the cell body in the brain, it was not until the observations of Coghill (*op. cit.*) that important and hitherto unknown relations of the axonal collaterals in the cord became known. Based on his studies, Mauthner's fibers are regarded as constituting an inhibitory system. Following his observations on the synaptic connections within the cord, Coghill says: "Upon the hypothesis that the action is inhibitory, a volley of impulses passing cephalo-caudal along Mauthner's fibers would inhibit local sensory-motor responses, represented by the cells of intercalated type, in favor of the total action pattern and at the same time would inhibit the antagonist of one side in favor of the agonist of the other, as represented by the cells of motor type. Such inhibition would facilitate the cephalocaudal progressing flexures which effect swimming. Possible interference of local patterns of action (reflexes) with the total action pattern of swimming would thus be prevented, and interference of the axial musculature of the right side with that of the left and vice versa, would be blocked."

Coghill's interpretations are in harmony with my own findings based upon the excision of these cells (Detwiler, 1927, 1933), where it was shown that the swimming reflexes of larvae lacking one or both neurones are inferior to those of normal animals. Larvae devoid of one Mauthner's neurone were found to exhaust more quickly than normals following repeated tactile stimulations. The swimming frequently was jerky and uncoordinated. There was no evidence from these experiments that the lack of one of the Mauthner's neurones had any effect upon equilibration.

In excising a Mauthner's cell, the ear vesicle first had to be removed and subsequently replaced by a vesicle from a donor embryo. Histological studies showed that, when larvae lacking the Mauthner's cell exhibited equilibratory disturbances, they were referable to an abnormal ear or to defective nervous connections, or both. It became apparent, from the results, that Mauthner's neurones are not indispensable to normal equilibrium. On the other hand, the absence of one ear was followed by marked equilibratory disturbances.

According to Coghill's view, the removal of Mauthner's fiber would release a normally present inhibitory effect on the local patterns of action and the interference between this and the total action pattern would be increased, as well as that between the axial musculature of both sides of the body. Regardless of the exact way in which the mechanism works, both the histological and physiological findings agree that this system is important in sustaining coordinated motor activity.

When the earlier experiments were made upon Mauthner's cells, no device was available for measuring the motor responses. It seemed desirable, therefore, to repeat the experiments and test their locomotor responses with the device shown in FIGURE 7.

The technique employed in removing the Mauthner cell consisted in first excising the ear vesicle. A small mass of cells was then cut away from the lateral wall of the medulla just medial to the ear region. This was followed by replacement of an ear vesicle from a donor embryo, using care that it was implanted with normal orientation. The operation was made on embryos in the early tail-bud stage (stages 27 and 28). For studies on comparative behavior, the ear vesicle alone was removed from a group of similar aged embryos, leaving the Mauthner neurone intact. In both groups, the *C* and *S* flexures and the early swimming responses were carried out in normal fashion. Beginning with stage 38, the motor responses were measured upon three groups as follows: (*1*) 10 normal control larvae; (*2*) 20 larvae from which the right Mauthner's cell had presumably been removed; and (*3*) 10 larvae lacking the right ear vesicle. This was done with the device shown in FIGURE 7. Each animal was stimulated 25 times, at approximately 5-second intervals, by stroking the trunk skin with a human hair as described above. The total number of units (sectors of arc) traveled by each animal following 25 stimulations was then computed, the average determined for the individuals in each group, and the standard error computed.* This procedure was carried out for each successive stage up to the feeding stage (44+) and then upon larvae 2, 4, and 7 days respectively after feeding.†

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* I am indebted to Professor Herbert Elftman for subjecting my data to a statistical analysis and computing the standard error.

† In *A. punctatum*, the larvae begin to feed at stage 46. In these experiments, *A. jeffersonianum* was used and they were observed to begin feeding at a developmental period comparable in many respects to stage 44 of *A. punctatum*.

The data, based upon approximately 10,000 individual recordings, are given in FIGURE 24. An examination of this figure reveals a number of interesting facts. The larvae of all three groups made relatively low scores at stage 38, those lacking the ear being the lowest. At stage 39, there was a rise in the scores of all groups, but the controls markedly outdistanced those of the other two groups. Here, again, those lacking the ear made the poorest records; those lacking Mauthner's neurone occupied an intermediate position. From stage 40 on, there was a general overall decline in the motor ability of the group with theoretical absence of the Mauthner's neurone. Although there existed considerable variation at different stages, those lacking the ear showed marked improvement in their scores after stage 44, and at 4 days after the feeding stage their scores equaled those of the controls. Despite the improvement in their locomotor ability, these larvae lacking the ear still exhibited the characteristic equilibratory disturbances during locomotion. The behavior of larvae lacking an ear vesicle has been described by Greene and Laurens (1923) and subsequently by the author (1927, 1933). Such animals exhibit torsion towards the earless side. There is flexion of the arm on that side, with extension of the opposite arm. In swimming, the larvae rotate frequently on the longitudinal axis so as to exhibit a "corkscrew" movement. They may come to rest upside down, but more frequently land on the earless side. Spontaneous movements are usually less marked.

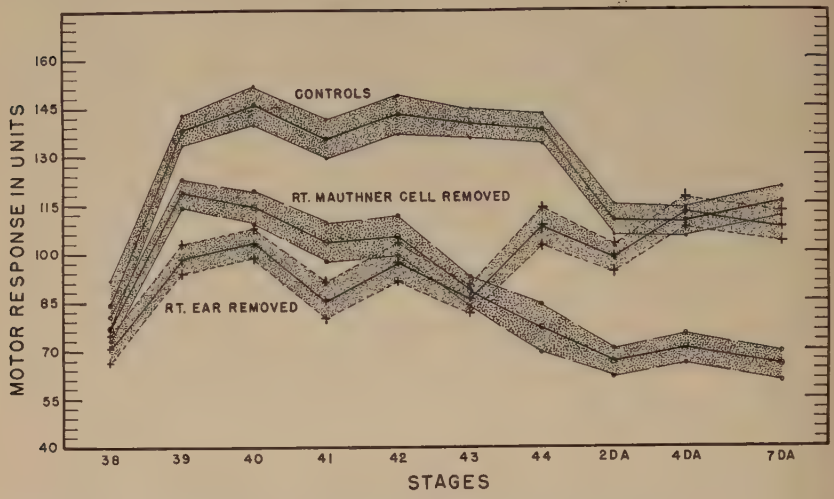

FIGURE 24. Graph showing average total distance traveled by larvae of *A. jeffersonianum* in response to 25 successive stimulations at 5-second intervals under the conditions indicated. The heavy central line for each curve represents the average; the line above and below represents the standard error. The curves are based upon the data obtained from 10 normal larvae, 20 with the right Mauthner's cell removed, and 10 with the right ear removed. Upon later histological examination, it was found that in 7 of the Mauthner neurone series the cell had not been excised (*v.* text for explanation).

The larvae with theoretical absence of Mauthner's neurone showed no signs of improvement in their scores up to 7 days *post* feeding. Incomplete records of some older larvae of this group showed improvement in locomotor response, but the data were too scattered to be treated statistically. Viewing the curve as a whole, it is apparent that larvae lacking Mauthner's cell do not have the locomotor capacity of normal larvae. The fall in locomotor ability of this group began at stage 39. It may be pertinent to point out from microscopic studies of normal larvae that, at stage 39, Mauthner's cell shows some differentiation, which appears to be complete by stage 41.* According to the records (FIGURE 24), it is at this period when the motor response of this group began to decline (*cf.* controls).

In order to obtain evidence as to whether the Mauthner neurone had actually been excised, the larvae of this group were studied in serial transverse section. Whereas the group was originally made up of 20 larvae, only 16 were available at the end of the experiment. Both Mauthner's neurones were found to be present in 7 of the 16 animals. Most of these individuals, from which the right neurone had not been excised, also had low scores, but the average, on the whole, was higher than that of the group lacking this neurone. The low scores of the group with this neurone intact, as compared with those of the controls, leads one to suspect that, whereas the cell had not been excised, the operation may have disturbed the relations sufficiently to prevent normal connections of the cell body, resulting thereby in a lack of normal function. This theoretical explanation received some support from the fact that, in several cases, the perikaryon was displaced and in 2 cases was very small.

This series of experiments is regarded as incomplete, and it is planned to repeat the experiment and test the locomotor responses of much older larvae along with those of normal controls of similar age. In so far as they go, the results bear out former conclusions that the locomotor ability of larvae lacking the Mauthner fiber is inferior to that of normal animals.

Summary and Conclusions

1. With a device described in the text (FIGURE 7), the locomotor responses of young *Amblystoma* larvae have been quantified under the following conditions:

(*a*) Control larvae for each group listed below.

(*b*) Larvae lacking the cerebral hemispheres and the dorsal portion of the diencephalon.

(*c*) Larvae with the midbrain replaced by the anterior end of the spinal cord.

(*d*) Larvae with end-to-end reversal of the mesencephalon.

(*e*) Larvae with unilateral reversal of the mesencephalon (reversal of A–P axis only).

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* Based upon a microscopic study of Harrison's normal stages.

(*f*) Larvae lacking the right Mauthner's neurone, but with intact ears.

(*g*) Larvae lacking the right ear.

B. The data obtained from this group, where locomotor responses were measured from stages 39 to 46+, show that during these stages the motor capacity is only slightly lowered (FIGURES 16 and 17). The relative unimportance of the hemispheres in the general motor activities is clearly indicated.

C. Larvae without the midbrain, but with nervous continuity between fore- and hindbrain exhibit normal locomotor responses up to approximately stage 40. Thereafter, the motor capacity is greatly lowered (FIGURES 9 and 11). The data indicate that the swimming mechanism in the early stages is essentially spinal and autonomous, but that at approximately stages 40–41 it becomes subservient to mesencephalic control. probably concomitant with the development of the tecto-bulbar and tecto-spinal tracts.

D. Reversal of the mesencephalon only slightly lowers the locomotor capacity. The scores of larvae ranging from stages 39 to 46+ are in general of a magnitude similar to those exhibited by larvae lacking the hemispheres (*cf.* FIGURES 13 and 16).

E. Unilateral reversal of the midbrain (A–P axis only) has no effect upon the motor ability of the animal.

F. Animals lacking one Mauthner's neurone show significantly lowered scores as compared with those of control larvae (FIGURE 24). They differ from those lacking an ear in that the scores did not improve in the later stages under investigation.

G. The lowered motor ability of larvae lacking one ear is rather striking up to near the feeding stage. Thereafter, there is a gradual improvement. Four days after the feeding stage their scores equaled those of normal larvae (FIGURE 24).

The data obtained upon all groups are listed in graph form in the text. The method of study, as presented, gives an objective and quantitative picture of the performance of young larvae under various experimental conditions. It makes possible statistical validation of small differences which might be in doubt if reliance were placed solely upon subjective impressions.

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FUNCTIONAL POLARIZATION IN DEVELOPING AND REGENERATING RETINAE OF TRANSPLANTED EYES*

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STUDIES of the organization and location of the eye-forming centers in the brain wall (Adelmann, 1934, 1936, 1937; Alderman, 1935; Mangold, 1931; Stone and Dinnean, 1943) and the subsequent growth of those centers related to the induction and organization of the lens as shown by Spemann (1938) and many others, reveal how well this sense organ is adapted in amphibians as a tool for approaching problems dealing with polarization during development and differentiation.

The influence of the developing eye cup upon the formation of the medio-lateral axis of the lens has been intimated by the experiments of Woerdeman (1934) and LeCron (1907), but little notice has really been given to it. The outer pole of the developing lens forms the subcapsular epithelium. The inner pole is devoted to fiber formation. The evidence offered by LeCron and Woerdeman seems to indicate that the establishment of this axis may be normally dependent upon the length of time the lens anlage remains in contact with the optic vesicle and cup. When the lens placode was isolated early from the optic vesicle, it formed later only an epithelial vesicle. If the lens anlage was allowed to be associated with an optic cup for a period of time before it was isolated, it could later develop a fiber-forming pole. Even in some of these cases, the fibers would degenerate later. The optic cup is probably exerting a strong influence upon the establishment of this axis. There is a great need for further study of this problem.

Other organization within the lens can also be studied. Early on the anterior (distal) surface of the lens is a vertical suture and on the posterior (proximal) surface is a horizontal one. Focusing attention on the posterior (horizontal) suture, Woerdeman (1934) found that, by rotating the presumptive lens ectoderm 90°, this suture was in a labile form before the period when the neural folds became elevated, but it was determined just before the neural folds closed. Therefore, the polarity or growth direction of the fibers was already predestined at an early stage. According to Woerdeman, this is also borne out by the fact that, when the optic vesicle is rotated 90°, the suture line in the lens is unrotated, although the choroid fissure was rotated.

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Since the direction of the choroid fissure normally coincides with that of the anterior vertical suture of the lens, and is at the same time perpendicular to the posterior horizontal suture, this relationship could be used in further fruitful studies to uncover any influence the eye-forming centers may be exerting on the establishment of the lens sutures.

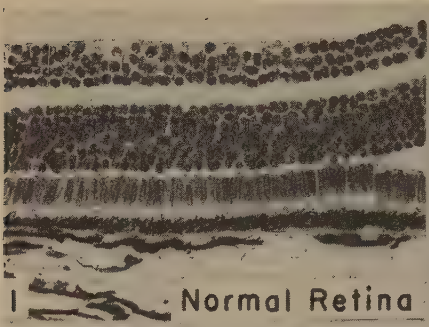
From the work of Beckwith (1927), Sato (1933), and Woerdeman (1934), one would judge that the time when the choroid fissure itself becomes determined varies in different species of amphibians. Since it can be used as a mark of polarization taking place in the eye cup, and since it marks the groove along which the early *lentis vasculosis* is directed in the higher vertebrates, it is highly desirable that the choroid fissure be examined even more closely than it has been in previous studies.

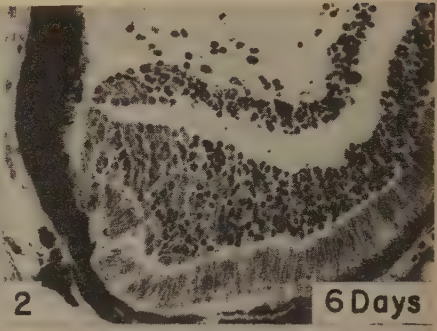
In the midst of all of these influences for organization, it may occur to the curious-minded that the future functional arrangement in the retina might also be manifesting itself. Therefore, as an interesting challenge from the standpoint of development and regeneration, we shall examine what evidence we have of this example of polarization taking place in the salamander eye.

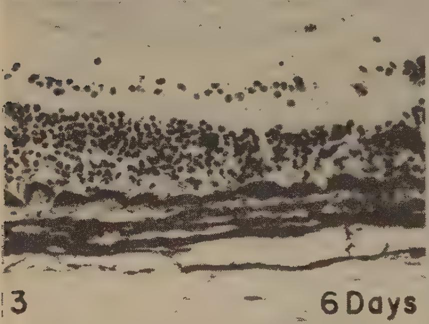
I have worked for many years on vision in salamanders, where the eye can be successfully transplanted followed by return of vision in the graft. Up to the present time, this has not been done successfully in other vertebrates. We have shown not only that the eyes of larvae (Stone, Ussher, and Beers, 1937; Stone and Cole, 1931 and 1943; Stone and Zaur, 1940; Stone, 1940) and adults can be transplanted to new hosts, and eventually vision returns in the graft, but that the eyes of quite different species of salamanders can be exchanged with equal success (Stone, 1930; Stone and Ellison, 1940 and 1945). This, I might say in passing, has presented an interesting method of studying visual acuity when it is different in two hosts whose eyes are being exchanged. Return of vision can be shown at least four times in the same adult salamander eye (*Triturus viridescens*) repeatedly transplanted to new hosts (Stone and Farthing, 1942). The adult salamander eye can even be successfully transplanted after seven days of refrigeration (Stone, 1946).

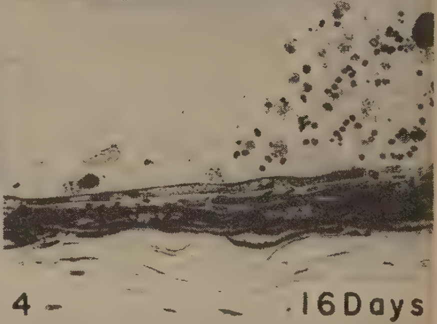
When the eyes are transplanted in larval salamanders, very little structural changes take place in the graft (Stone, Ussher, and Beers, 1937; Stone, 1930). The original retina is retained with an occasional loss of a few ganglion cells. The proximal stump of the optic nerve regenerates readily and follows along the pathway of the degenerated distal segment leading into the brain.

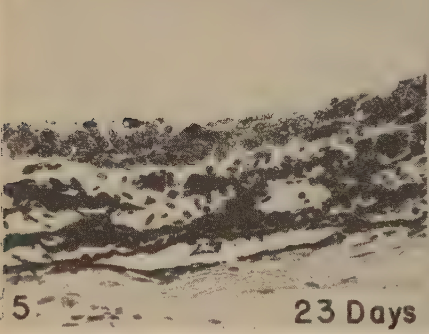
In the adult grafted eye of the salamander, a striking difference occurs. Not only all of the optic nerve degenerates but the original retina of the eye also disintegrates quite rapidly, with the exception of a ring of cells at the peripheral or ciliary margin (FIGURES 1–6). From these surviving cells, a new retina is regenerated and, after differentiation takes place, a new optic nerve grows back through the chiasma into the brain, where it apparently makes proper connections. Return of vision is usu-











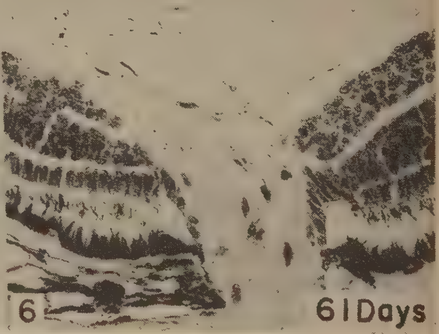


FIGURE 1. Photomicrograph of central portion of normal retina in adult eye of *Triturus viridescens.* x125. All other figures are from transplanted adult eyes of the same species.

FIGURE 2. Ciliary region in a transplanted eye 6 days after operation, showing far less degeneration than in the central retina (FIGURE 3). x125.

FIGURE 3. Same case as FIGURE 2, showing extensive degeneration in the central retina 6 days after operation. x125.

FIGURE 4. Showing complete degeneration of the central retina 16 days after operation. x125.

FIGURE 5. Showing a regenerating retina, three to four cells deep, in the central portion 23 days after operation. x125.

FIGURE 6. Showing the completely regenerated retina and optic nerve 61 days after operation. x125.

ally demonstrated in the transplanted eye between two and a half and three months after operation.

When Sperry, in 1942, first reported reversed visuomotor responses in adult salamanders after optic nerve regeneration in eyes which were rotated *in situ* without removal from the orbit, I examined the effects of various types of rotation on vision in many grafted eyes of *Triturus viridescens* in which the regenerated and new functioning retina could be tested (Stone, 1944). We shall return to these experiments later.

Since we shall be examining the functional quadrants of the retina, we need to know the normal visuomotor responses in the salamander, which are both simple and dependable for our tests. They are demonstrated when the animal moves toward, snaps at, and follows a lure approaching any of the four quadrants in the field of vision. The animal also automatically moves head and body in the same direction with a rotating black and white striped drum only if it passes from the temporal to the nasal poles (postero-anteriorly) through the field of vision of one eye (clockwise for a left eye and counterclockwise for a right eye). These compensatory movements are not elicited when the drum moves in the nasotemporal or antero-posterior direction through the field of vision of either eye.

In attempting to determine the stage in development at which the retina becomes functionally polarized, the right eye was excised, rotated 180°, and reimplanted in embryos of *Amblystoma punctatum* from the closure of the neural folds to a period just before the feeding stage begins. In other words, all the Harrison stages from 20 to 44 inclusive were examined. Vision tests during the larval and adult life of these hosts showed that, up to the late tail-bud stages, the eye could be rotated 180° without normal vision being affected later.

If the eye is rotated around the stage at which the first motor responses are known to appear in the embryo, the visuomotor reactions in the animal later on appear to be confused. Reactions sometimes appear slightly reversed but are not sharp and consistent. It is difficult to analyze them. However, if the eye is rotated at about the time when the beating of the heart is prominent, around Harrison stage 36, a definite reversal in the visuomotor responses will later become evident. The eye is now developed into a well-defined, broad, smooth-rimmed cup. A thin tapetum covers the outer surface of the thick undifferentiated future retina. The lens vesicle is just separating from the source of origin and will soon produce a fiber-forming pole. It fills the cavity of the optic cup against which it has been very tightly pressed ever since it sank inward as a lens plug. Anyone who has tried to remove the living lens anlagen at this time will know full well the implications of these remarks as applied to the operation. If the degree of adhesion with which they stick together is any indication of the importance of events now happening between them before they later separate, it must be all-powerful. In fixed preparations, this relationship is poorly indicated as a distinctive feature. I believe it is worthy of far more attention than has been placed upon it.

Leading up to this period in development, the functional polarization of the retina is becoming established. Not long after this critical period, all rotated eye cups show that later in larval and adult hosts both the dorsoventral and antero-posterior (nasotemporal) axes are fully expressed by a complete reversal of the vision responses. Whether both axes are established at the same time or at different moments is being investigated by experiments now in progress.

In an earlier part of this discussion, attention was called to the fact that, as soon as one temporarily interferes with the blood supply to the retina in an adult salamander eye, such as transplantation accomplishes, the retina degenerates with the exception of a ring of cells at the peripheral or ciliary margin. These surviving cells aid in the regeneration of a retina which gives off a new optic nerve. This grows out to make connections with the brain, so that return of vision can be demonstrated between two and three months after operation. This offers an unusual opportunity not only to examine the possibility of return of vision but to test whether or not the functional quadrants in the retina can be re-established through the processes of regeneration.

In my studies on many hundreds of transplanted eyes in salamanders, it has been shown that normal vision eventually returns to the graft if it is normally oriented when placed in the orbit. In some manner, the new fibers from the ganglion cells in each of the quadrants in the regenerated retina in adult transplanted eyes register their stimuli with the proper centers in the optic tectum. It is difficult to conceive how normal vision could be re-established if this were not so.

To examine further the functional quadrants of the retina in the adult eye of *Triturus viridescens*, for example, we must perform a few simple operations involving various types of rotation experiments. In the first of these experiments, we shall rotate the right eye 180° anterodorsally *in situ* and fix it in place after cutting only the conjunctival and muscular attachments. Care is taken to insure that the optic nerve and blood supply are left intact in order to preserve the original retina. To prepare our animal for special tests, we shall remove the opposite left, normal eye. Following our usual technique, the animal, remaining under chloretone anesthesia, is placed in a cool moist chamber where it continues quiet for 24 hours. During this time, the right rotated eye becomes fixed and healed in place. Animals prepared in this manner are now placed in water in finger-bowl aquaria where they are kept for daily observations. We shall see that the eyes now possess all the quadrants of the original intact retinae functionally reversed.

The animals have a tendency to swim and walk in circles, sometimes in a very excited manner in short circles in the center of the aquaria with the head touching the tail. Their circuitous progression is almost entirely toward the blind side and, as they come to rest, the head continues to drift for a short distance in the direction in which they have been moving before it is brought back to the midline. When *Daphnia* are swimming about in the aquaria, these salamanders make erroneous strikes at the moving objects. Unless they make contact with their source of food, they may have great difficulty in obtaining it.

In response to a rotating black and white striped drum, the head and body movements are called forth only when the drum passes through the field of vision from the original temporal (posterior) pole to the original nasal (anterior) pole. Since the right eye has been completely rotated 180° in this case, the drum must be moving in the clockwise direction—the one which fails to call forth a reaction if the right eye is normally oriented. Instead of following in the direction of the moving drum, however, the animal walks or swims in the opposite or reverse direction. At first, the head starts drifting in the opposite direction. After reorientation, this nystagmoid movement may be repeated several times. Very soon the animal starts swimming and walking as if pursuing the drum, but always in the reverse direction. When the drum is rotated in the opposite direction (counterclockwise) through the field of vision from the original nasal (anterior) pole to the original temporal (posterior) pole, the animal usually assumes the position of a fixed stare. There is no nystagmoid movement of the head or body, and the animal makes no movements in response to the drum.

A dark object, such as a small piece of red rubber impaled on the end of a white wire, serves as a good lure for further vision tests. When the lure is brought from in front into the field of vision, the animal immediately seeks it in the opposite direction. When the lure approaches from the rear, the animal moves forward in its search. When the lure is brought above the animal, it immediately darts to the bottom of the aquarium to find the moving object, and when the lure is moved below the glass bottom of the aquarium the animal comes to the top of the water in its pursuit. In other words, all responses are completely reversed. If the animal is anesthetized again and the eye rotated back through the same arc to normal orientation, the swimming reactions and all visuomotor responses to the lure and drum are fully restored to normal as soon as the animal recovers from the anesthetic. We now have sufficient control observations to compare with the results obtained from rotated transplanted eyes with return of vision in a regenerated retina that replaces the original one.

Our next experiment, then, will be to excise a right eye, for example, and then reimplant it upside down. We have then rotated all retinal quadrants of the eye 180°, just as we did in the above experiment when the eye was rotated 180° *in situ* without removing it from the orbit. The general technique of operation is the same as that which I have often described before in the literature already cited. Since the degeneration and regeneration of the retina and optic nerve and return of vision will be completed at the end of two or three months, we must excise the left normal eye before that time, in order to make our tests comparable to the one above, that is, exclusively on the rotated eye. We find that, as soon as vision has returned, all of the visuomotor responses are as completely reversed as in the case where the eye was rotated in the orbit without destroying its original retina. If the eye is now rotated 180° *in situ* so that its axes are normally oriented, this places the retinal quadrants in normal position, and the visuomotor responses are normal again. Also, if a reimplanted rotated (180°) eye, which has been functioning for a considerable period with reversed vision, is transplanted a second time but normally oriented, the swimming reactions of the animal and the visuomotor responses are perfectly normal in every respect when vision returns again in the retina which regenerates for the second time.

We can study, in the same eye, the effects of rotation on two quadrants of the retina while the other two remain normally oriented (Stone, 1944; Sperry, 1945). This is done in two simple experiments, by excising either the right or left eye and grafting it to the opposite side. If, for example, we excise both eyes, discard the right one and, in its place, implant the left eye without inverting the dorsoventral axis, the quadrants in this axis are normally oriented but the antero-posterior (nasotemporal) axis is rotated 180°, thus reversing only the positions of the nasal (anterior) and temporal (posterior) quadrants. When vision returns in the new retina, the swimming, head movements, and reactions to a rotating drum are the same as when this axis (antero-posterior) was rotated in the eyes reimplanted upside down. Reactions to the lure were completely reversed and abnormal when the lure approached from in front or from the rear, but perfectly normal when it approached either dorsally or ventrally.

In another experiment, if we excise both eyes, discard the right one and, in its place, implant the left eye upside down this time, we maintain the normal orientation of the anterior and posterior quadrants but invert both the dorsal and ventral quadrants. Now, when the vision returns, if the lure is brought into vision above the water in the aquarium, the animal immediately seeks it at the bottom of the aquarium and *vice versa*. In seeking the lure moving into vision from the front or from the rear, the visuomotor responses were perfectly normal. Since this axis (antero-posterior) was not rotated, the head and body movements, swimming, and reactions to the rotating drum were the same as in animals with a single normally oriented right eye. Therefore, we can prove that the functional patterns in each of the retinal quadrants are re-established by regeneration and that the visuomotor responses are guided by the orientation which these retinal quadrants register to the central nervous system. How the peripheral and central connections are properly made to maintain these abnormal vision responses for over three years, is still a mystery and there is, as yet, no good evidence recorded in the literature to help us solve it.

There are other recorded studies of vision responses in amphibians following rotation of functional eyes. Some of the procedures of operation have been carried out in a similar manner and others by a method different from what I have already described. Sperry, in 1943, gave a detailed account of the effects on vision after the adult eye of *Triturus viridescens* was rotated 180° *in situ*, preserving the original retina and optic nerve. Concerning abnormal swimming reactions, drifting head and body movements, the reversed reactions to the moving lure and the rotating drum, my observations corroborate his findings. To determine whether or not the regenerating optic nerve in a 180° rotated eye would connect with the brain in an orderly fashion to show the same abnormal pattern of vision, Sperry (1943a) rotated eyes 180° *in situ*, allowed them to heal in place, and then severed the optic nerve. In some, the cut ends of the nerve were twisted or crushed to increase all chances for confusion as the nerve fibers grew back to the chiasma and brain. In some cases, the blood supply to the retina was apparently not disturbed and the optic nerve must have regenerated without retinal degeneration such as Stone and Chace (1941) found. From his description of the gross appearance of some of the eyes and delay in return of vision, the blood supply to the retina must have been severed along with the optic nerve, in which case the retina was replaced after degeneration. In any event, all of his cases showed vision completely reversed, as in the case of those eyes which, as I mentioned, were reimplanted upside down.

Although one cannot transplant eyes of tadpoles or adult anurans successfully, because the retina will not regenerate (Stone, 1938 and 1940), the optic nerve will regenerate when cut if the blood supply to the retina remains intact. However, Sperry (1944) was able to extend his types of operations to larval and adult anurans by rotating the eye *in situ* and then cutting the optic nerve. The same effects on the visuomotor responses occurred in these amphibians as had been noticed in the salamanders.

Sperry also made another very interesting experiment. It is well known that, in amphibians, all the optic nerve fibers from an eye pass to the contralateral side. By destroying the optic chiasma and directing the proximal stump of the optic nerve to the pathway of the distal stump on the same side, Sperry (1945) forced the regenerating optic fibers in adult anurans to enter, on the ipsolateral side, the optic tectum with which it never had been and never is normally connected. Peculiar circus movements and mixed reverse reactions and rotating movements of the head and body, accompanied by ocular nystagmus, resulted after vision returned. The animals made errors in spatial localization when striking at a moving object or in trying to escape from an approaching large object. Further study is needed to understand these interesting reactions.

Our objective was to examine evidence dealing with the polarization of the functional quadrants of the retina. However, I think it has become obvious that the development of polarization within the optic tectum is a very intimate part of this general problem. To unravel it will be no small challenge to our ingenuity.

Summary and Conclusions

(*1*) Attention is called to the early polarization taking place in both the lens and the optic vesicle and to the interrelating influences being expressed between them at this time.

(*2*) 180° rotation of many stages of embryonic eyes in *Amblystoma punctatum* demonstrated that functional polarization of the retina is taking place in the optic cup of embryos shortly after the first motor responses appear.

(*3*) Function was also studied in the retinal quadrants as it was reestablished in regenerated retinae of rotated, grafted eyes of adult salamanders. In some cases, both the dorsoventral and nasotemporal (anteroposterior) axes were reversed (eyes reimplanted upside down). In others, one axis was reversed while the other remained normal (right and left eyes exchanged and properly rotated 180° on the one or the other axis).

(*4*) Complete reversal of visual perception occurred only in the rotated retinal quadrants, as exhibited by the visuomotor responses to a moving lure and to a rotating black and white striped drum.

(*5*) The reversed vision is permanent and only restored to normal when the eye is rotated back to normal orientation.

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